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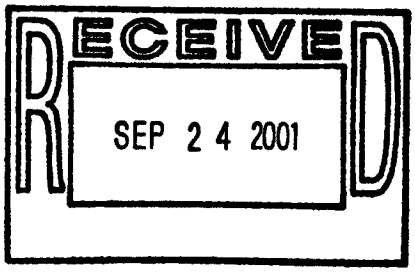
September 24, 2001



Via E-Mail and Federal Express

Dr. C.W. Jameson
National Toxicology Program
Report on Carcinogens
79 Alexander Drive
Building 4401, Room 3118
P.O. Box 12233
Research Triangle Park, NC 27709

Re: National Toxicology Program: Call for Public Comment on 16 Substances, Mixtures and Exposure Circumstances Proposed for Listing in the Report on Carcinogens, Eleventh Edition: 66 Fed. Reg. 38430 (July 24, 2001)

Dear Dr. Jameson:

The Alkanolamines Panel (Panel) of the American Chemistry Council submits the appended comments in response to the National Toxicology Program's (NTP) call for comments on the proposal to list diethanolamine (DEA) in the Eleventh Edition of the *Report on Carcinogens* (*RoC*). 66 Fed. Reg. 38430 (July 24, 2001). The attachments to the comments will be sent via Federal Express. Please direct any questions that you might have concerning these comments to Mr. Jonathon T. Busch, Manager of the Alkanolamines Panel, at (703) 741-5633.

Sincerely yours,

Signature [redacted]

Attachment

BEFORE THE NATIONAL TOXICOLOGY PROGRAM

COMMENTS OF THE ALKANOLAMINES PANEL OF THE AMERICAN CHEMISTRY COUNCIL IN RESPONSE TO NTP'S REQUEST FOR COMMENTS ON THE NOMINATION OF DIETHANOLAMINE FOR POSSIBLE LISTING IN THE *REPORT ON CARCINOGENS*

National Toxicology Program; Call for Public Comments on 16 Substances, Mixtures and Exposure Circumstances Proposed for Listing in the Report on Carcinogens, Eleventh Edition; 66 Fed. Reg. 38430 (July 24, 2001)	))))))

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EXECUTIVE SUMMARY

The Alkanolamines Panel (Panel) of the American Chemistry Council submits these comments in response to the National Toxicology Program's (NTP) call for comments on the proposal to list diethanolamine (DEA) in the Eleventh Edition of the *Report on Carcinogens* (*RoC*). 66 Fed. Reg. 38430 (July 24, 2001). The Panel members include major manufacturers of alkanolamines, including producers of DEA. In addition, the Panel supports and incorporates by reference the comments separately submitted by the Cosmetic, Toiletry, and Fragrance Association (CTFA).

DEA has been nominated for listing in the *RoC* based on the results of an NTP bioassay that reported clear evidence of carcinogenicity in male and female $B6C3F_1$ mice. For the reasons provided below, neither this bioassay, nor, to the Panel's knowledge, other evidence, provides a basis for listing DEA under NTP's "reasonably anticipated to be a human carcinogen" listing criteria. Specifically, there is insufficient evidence of carcinogenicity either in humans or from studies on experimental animals to conclude that DEA is "reasonably anticipated to be a human carcinogen" under the NTP criteria for listing in the *RoC,* and no other supplementary data meet the listing criteria.

The Panel bases this conclusion on the following considerations:

- The NTP mouse bioassay on DEA does not support a determination that DEA is "reasonably anticipated to be a human carcinogen" under the NTP criteria for listing in the *RoC*, even if valid or relevant to human risk, because it does not indicate that DEA induced a combination of malignant and benign tumors at multiple tissue sites or to an unusual degree.

  - The NTP mouse bioassay does not indicate a significant increase in a combination of malignant and benign renal tubule neoplasms and hence, on those grounds alone, does not establish an increased incidence of malignant and/or a combination of malignant and benign neoplasms at multiple tissue sites.

  - Various biological and other factors indicate that no tumors were induced to an unusual degree in the NTP mouse bioassay. This conclusion is consistent with the International Agency for Research on Cancer's (IARC) findings in determining that DEA should be classified as a Group 3 chemical.

- The negative National Institute of Environmental Health Sciences (NIEHS) Tg.AC transgenic mouse study on DEA is inconsistent with the NTP DEA bioassay and further demonstrates that there is insufficient

evidence to justify listing DEA. The authors of the NIEHS Tg.AC transgenic mouse study state that the NIEHS Tg.AC study indicates that the conventional NTP mouse bioassay likely "has given a false positive result."

- The NTP mouse study should not be used as a basis for listing DEA in the *RoC* because of technical limitations that preclude it from constituting sufficient evidence that DEA is "reasonably anticipated to be a human carcinogen." These technical limitations include use of obese mice, use of ethanol as the vehicle for administration of DEA, use of doses that may have exceeded the maximum tolerated dose, and failure to take measures to prevent ingestion of the test material by grooming. These limitations confounded or otherwise compromised the validity of the results of the NTP mouse bioassay.

- The limited value of increased liver tumors in mice, as recognized by leading authorities, further supports the conclusion that the NTP study should not be used as a basis for listing DEA in the *RoC.*

- The NTP mouse bioassay does not support listing under NTP's criteria because mechanistic research specifically on DEA indicates that, to the extent DEA can potentially induce tumors in mice, it does so by a mechanism that is not relevant to humans.

- Extensive evidence shows that DEA is not genotoxic, and there is no other available corroborative evidence that would support a listing in the *RoC.*

- The NTP DEA condensate studies do not support the conclusion that DEA may "reasonably be anticipated to be a human carcinogen" and in any event, as IARC concluded, those studies may not be relied upon in evaluating the carcinogenicity of DEA.

- NTP should follow the lead of IARC and conclude that DEA is not classifiable as to its carcinogenicity to humans and hence may not reasonably be anticipated to be a human carcinogen under the NTP criteria.

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INTRODUCTION

The Alkanolamines Panel (Panel) of the American Chemistry Council submits these comments in response to the National Toxicology Program's (NTP) call for comments on the proposal to list diethanolamine (DEA) in the Eleventh Edition of the *Report on Carcinogens* (*RoC*). 66 Fed. Reg. 38430 (July 24, 2001). The Panel members include major manufacturers of alkanolamines, including producers of DEA.[1] In addition, the Panel supports and incorporates by reference the comments separately submitted by the Cosmetic, Toiletry, and Fragrance Association (CTFA).

DEA has been nominated for listing in the *RoC* based on an NTP Technical Report[2] which concludes that under the conditions of a two-year dermal bioassay on $B6C3F_1$ mice, there was clear evidence of carcinogenic activity of DEA in male and female mice. The NTP Technical Report bases this conclusion on increased incidences of liver neoplasms in males and females and an increased incidence of renal tubule neoplasms in males. The same NTP Technical Report finds that there was no evidence of carcinogenic activity of DEA in a separate two-year bioassay on male and female F344/N rats.

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1 Panel member companies are: BASF Corporation, The Dow Chemical Company, Equistar Chemical, L.P., Huntsman Corporation, and Ineos, L.L.C.

2 Reference [1]. (Reference numbers in brackets correspond to the references listed on the Reference List appended to these comments.)

For the reasons provided below, neither this bioassay, nor, to the Panel's knowledge, other evidence, provides a basis for listing DEA under the NTP listing criteria.

I. BACKGROUND INFORMATION

The NTP Technical Report reports on two-year chronic toxicology and carcinogenesis bioassays of DEA in F344/N rats and B6C3F$_1$ mice. In the rat bioassay, groups of 50 male rats were administered 0, 16, 32, or 64 mg DEA/kg body weight in ethanol dermally for two years and groups of female rats were administered 0, 8, 16, or 32 mg DEA/kg in ethanol dermally for two years. In the mouse bioassay, groups of 50 male and 50 female mice were administered 0, 40, 80, or 160 mg DEA/kg body weight in ethanol dermally for two years. The controls were administered the ethanol vehicle (95% ethanol). The NTP Technical Report concludes that under the conditions of these studies, there was no evidence of carcinogenicity of DEA in the male or female rats. It further concludes that there was clear evidence of carcinogenic activity of DEA in male and female mice based on increased incidences of liver neoplasms in males and females and increased incidences of renal tubule neoplasms in males.

A Tg.AC transgenic mouse study was also conducted by the National Institute of Environmental Health Sciences (NIEHS) on DEA.[3] Using a 20-week exposure protocol, homozygous Tg.AC mice were topically treated with DEA in a 95% ethanol vehicle. DEA was administered in this study at dose levels that significantly exceeded the highest dose used in the NTP mouse bioassay on DEA. The highest dose administered in the transgenic mouse study was

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[3] Reference [2].

20 mg DEA/mouse/day (800 mg/kg/day).[4] This study gave negative results for tumorigenic effects.

II. NTP REQUIRES THAT BEFORE A SUBSTANCE MAY BE LISTED IN THE *RoC*, THAT SUBSTANCE MUST BE DETERMINED TO BE "REASONABLY ANTICIPATED TO BE A HUMAN CARCINOGEN" UNDER SPECIFICALLY DELINEATED CRITERIA

Chemicals may be listed in the *RoC* if they are determined to be "known to be human carcinogens" or "reasonably anticipated to be human carcinogens."[5] The applicable criteria for listing are as follows:[6]

- Studies in humans indicate either: (1) there is sufficient evidence of carcinogenicity from studies in humans which indicates a causal relationship between exposure to the agent, substance, or mixture and human cancer ("known to be human carcinogen") or (2) there is limited evidence of carcinogenicity from studies in humans which indicates that causal interpretation is credible, but that alternative explanations, such as chance, bias, or confounding factors, could not adequately be excluded ("reasonably anticipated to be human carcinogen").[7]

- Sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors ("reasonably anticipated to be human carcinogen"):

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4 *Id.* at 218.

5 61 Fed. Reg. 50499-50500 (Sept. 26, 1996).

6 *Id. See also* 66 Fed. Reg. at 38430; NTP, *Report on Carcinogens, Ninth Edition, Carcinogen Profiles 2000*, at I-2.

7 DEA has not been nominated based on human studies, and as discussed below, there are insufficient human data to raise an issue as to whether DEA may be listed based on human studies.

- In multiple species or at multiple tissue sites; or
- By multiple routes of exposure; or
- To an unusual degree with regard to incidence, site, or type of tumor or age at onset.

- When there is less than sufficient evidence of carcinogenicity in humans or laboratory animals, a chemical may nevertheless be found to be "reasonably anticipated to be a human carcinogen" based on other considerations concerning structure and mechanism. For example, a substance may be listed if it belongs to a well-defined, structurally related class of substances whose members are listed in a previous *RoC* as either known to be a human carcinogen or reasonably anticipated to be a human carcinogen.

- Conclusions regarding carcinogenicity are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to, dose response, metabolism, and pharmacokinetics. Importantly, substances for which there is evidence of carcinogenicity in laboratory animals are not considered "reasonably anticipated to cause cancer in humans" where there are compelling data indicating that the agent acts through mechanisms which do not operate in humans.

For the reasons discussed below, neither the NTP two-year mouse bioassay, nor to the Panel's knowledge, any other data, meet the NTP criteria for listing.

III. THERE ARE INSUFFICIENT HUMAN DATA TO RAISE ANY ISSUE AS TO WHETHER DEA IS KNOWN OR IS REASONABLY ANTICIPATED TO BE A HUMAN CARCINOGEN

The nominating body for DEA, the United Auto Workers, does not base its nomination of DEA on any human data.[8] Further, to the Panel's knowledge no human studies

[8] *See* 66 Fed. Reg. at 38431.

raise any issue as to whether DEA should be listed. Finally, the NTP Technical Report on DEA states that no references to carcinogenicity in humans were found in a review of the current literature on DEA.[9]

IV. BECAUSE THE NTP TWO-YEAR RAT BIOASSAY ON DEA WAS NEGATIVE, THERE IS NO ISSUE AS TO WHETHER DEA HAS INDUCED TUMORS IN MULTIPLE SPECIES

As discussed above, the NTP Technical Report concludes that there was no evidence of carcinogenicity of DEA in the male or female rats in the two-year rat bioassay. Other than the NTP two-year mouse bioassay, there exist no other studies, to the knowledge of the Panel, that have reported an increased incidence of tumors. Accordingly, there is no basis for concluding that DEA induces tumors in multiple species (or by multiple routes of exposure) and therefore can satisfy the NTP listing criteria on those grounds.

V. THE NTP MOUSE BIOASSAY ON DEA DOES NOT SUPPORT A DETERMINATION THAT DEA IS "REASONABLY ANTICIPATED TO BE A HUMAN CARCINOGEN" UNDER THE NTP CRITERIA FOR LISTING IN THE *RoC*, EVEN IF VALID OR RELEVANT TO HUMAN RISK, BECAUSE IT DOES NOT INDICATE THAT DEA INDUCED A COMBINATION OF MALIGNANT AND BENIGN TUMORS AT MULTIPLE TISSUE SITES OR TO AN UNUSUAL DEGREE

Even if the NTP mouse bioassay were valid or relevant to humans, the study would not meet the fundamental NTP criteria for listing that requires a treatment-related

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[9] Reference [1] at 20.

increased incidence of a combination of malignant and benign tumors at multiple tissue sites or to an unusual degree.[10] Moreover, the NTP mouse bioassay is not valid or relevant to humans.

A. The NTP Mouse Bioassay, Even If Valid, Does Not Provide Evidence That DEA Induces Malignant or a Combination of Malignant and Combined Tumors at Multiple Tissue Sites[11]

The NTP mouse bioassay cannot reasonably be interpreted as establishing an increased incidence of malignant and/or a combination of malignant and benign tumors at multiple tissue sites. The only tumors that the NTP Technical Report concludes were increased in the mouse bioassay were benign and malignant liver tumors in both males and females, and renal tubule tumors (males only).[12] Contrary to the conclusion of the NTP Technical Report, the NTP mouse bioassay does not indicate an increase in a *combination* of *malignant* and benign *renal tubule* tumors, and hence does not establish an increased incidence of malignant and/or a combination of malignant and benign tumors, at multiple tissue sites, for the following reasons:

- There was no increase in malignant renal tubule carcinomas in the high dose group of male mice compared to controls *(i.e.*, the incidence was 2/50 in both the high dose group and the controls), based either on the

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10 Since the NTP mouse bioassay study involved only a single species and only a single intended route of exposure (dermal), that study by definition cannot meet the multiple species or multiple route of exposure criteria for listing. Moreover, as discussed above, there exist no other studies, to the knowledge of the Panel, that have reported an increased incidence of tumors.

11 As discussed below, the Panel believes that the NTP mouse bioassay is not valid and is not relevant to humans.

12 Reference [1] at 50.

single section data or the combined single and step section data.[13] There was in fact a negative trend in renal tubule carcinomas in the males from the controls through the mid-dose group.[14]

- There is no statistically significant ($p < 0.05$) increase at the high dose, nor in trend, of combined renal tubule adenomas and carcinomas (either after single section or after single and step sections). Indeed, while the incidence of the *benign* adenomas *alone* (after the extended analysis) is statistically significant at the high dose ($p=0.028$), the incidence falls to below the level of significance when combined with carcinomas ($p=0.055$).[15]

- The absence of an increased proportion of malignant tumors, in relation to benign tumors, let alone the absence of an increase in absolute numbers of malignant tumors, has been recognized by the U.S. Environmental Protection Agency (EPA) as an important factor to be considered in determining whether an increase in a combination of malignant and benign tumors is biologically significant.[16]

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13 *Id.* at 144 (Table C3).

14 *Id.* Contrary to the discussion in the NTP Technical Report (at page 50), the tabulated data at the end of the Report for the extended evaluation of kidney sections do not indicate the presence of an additional carcinoma in the high dose male group (160 mg/kg group). *Id.* at 144 (Table C3). Even if the tabulated data are incorrect with respect to the nature of the extra renal tubule tumor at the high dose and the extra tumor in fact was a carcinoma, an increase of only a single carcinoma in the high dose should not be considered of biological significance, particularly given that the increase of combined adenomas and carcinomas was not statistically significant at the high dose or in trend, as discussed below. The lack of biological significance is also indicated by the fact that the incidence of carcinomas remained at 0 in the mid dose after the extended analysis, compared to an incidence of two in the controls.

15 *Id.* at 144 (Table C3). The trend analysis for adenomas or carcinomas combined after single section and after the extended analysis gave $p=0.064$ and $p=0.056$, respectively.

16 *See, e.g.,* EPA, Draft Guidelines for Carcinogen Risk Assessment (July 1999) at 2-27; *see also* EPA, Proposed Guidelines for Carcinogen Risk Assessment, 61 Fed. Reg. 17960, 17976-17977 (Apr. 23, 1996).

B. The NTP Mouse Bioassay Does Not Indicate That Malignant or a Combination of Malignant and Benign Tumors Were Induced to an Unusual Degree with Regard to Incidence, Site, or Type of Tumor or Age at Onset

Because there was no increase in a combination of malignant and benign renal tubule tumors of any biological significance, such tumors were not induced to an unusual degree.

In addition, the incidences of hepatocellular carcinoma and/or combined hepatocellular adenoma or carcinoma in the treated mice, particularly in the females, were not increased to an "unusual degree," as other bodies considering the issue have similarly concluded. For example, in determining that DEA should be classified as a Group 3 chemical, the International Agency for Research on Cancer (IARC) concluded that DEA did not induce any tumors to an "unusual degree." NTP should conclude, as IARC did, that DEA did not increase hepatocellular tumors in female mice, or any other tumors in male or female mice, to an "unusual degree" in the NTP DEA study, for the following reasons: [17]

[17] Support for this conclusion and the reasons discussed below is provided largely in the appended letter from Dr. Gordon Hard, American Health Foundation, to Jonathon T. Busch, American Chemistry Council (Sept. 14, 2000) (Hard Letter) (Attachment 1) and references cited therein, and in William T. Stott, Ph.D., "Diethanolamine: A Conversation With OEHHA Staff" (Undated) (same as a document handed out at the Alkanolamines Panel's September 18, 2000, meeting with Office of Environmental Health Hazard Assessment (OEHHA) staff, except for minor technical corrections) (Stott Outline) (appended as Attachment 2), and the references cited therein. Additional supporting documentation is also cited in the footnotes accompanying the discussion below. Dr. Hard, a pathologist with the American Health Foundation, was also an active member of the IARC Working Group that evaluated DEA. Dr. Hard's role in the Working Group was Chairman of the Subgroup on Experimental Carcinogenicity Data. Dr. Stott is a research toxicologist with The Dow Chemical Company.

- Pathologists with expertise in rodent liver carcinogenesis have long accepted that liver tumors in rodents develop in a morphologic sequence of lesions along a continuum, with liver adenomas preceding and being able to progress into carcinomas.[18]

- The incidence of liver tumors in the vehicle (*i.e.*, ethanol-treated) control female and male mice in the NTP DEA study was unusually high, *i.e.*, the "baseline" incidence of liver tumors in mice not treated with DEA was high.[19]

- A tumor promoter would be expected to increase both the number and severity of the "baseline" tumor incidence and type.

- Where the incidence of hepatocellular adenomas in mice is high in the controls, as in the case of the NTP study, exposure to chemicals with only weak promotional potential can result in a high incidence of hepatocellular carcinomas.

- Due to its genetic predisposition to develop liver tumors, the $B6C3F_1$ mouse has an unusually high susceptibility to chemical-induced tumor formation and the $B6C3F_1$ carcinogenesis model cannot distinguish between promoters and true carcinogens.

- Based on the above considerations, IARC concluded that the NTP DEA bioassay did not provide "sufficient evidence," but rather only "limited evidence," of carcinogenicity in experimental animals.[20]

- Ethanol causes the loss of available choline in liver cells, thus exacerbating the choline depleting effects of DEA in rodents,[21] which are

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18 Consistent with this widely accepted view, Dr. J. R. Hailey of NTP commented that the hepatoblastoma observed in the NTP mouse study on DEA "appears to be part of the spectrum of the progression of liver neoplasms in the mouse; as such, with the higher background rate of liver neoplasms in mice, there is a concomitant increase in the incidence of hepatoblastoma." *See* Reference [1] at 12.

19 The incidence of tumors in ethanol-treated controls was: 66%, 64%, and 10% for adenomas/carcinomas combined, adenomas, and carcinomas, respectively, in females; and 78%, 62%, and 24% for adenomas and carcinomas combined, adenomas, and carcinomas, respectively, in males. Response in the female controls was outside the NTP historical control incidences. *See* Stott Outline, Table A; Reference [1] at 43 (males), 44 (females).

20 IARC (2000). *Monograph on the Evaluation of Carcinogenic Risks to Humans, Some Industrial Chemicals Monograph,* Vol. 77 at 372-374.

described below. Choline depletion in rodents can promote liver tumors.[22] Thus, the ethanol vehicle used in the NTP study for administering DEA is a confounding factor in interpreting the results and in and of itself precludes an "unusual degree" finding. [23]

VI. THE NEGATIVE TRANSGENIC MOUSE Tg.AC STUDY ON DEA IS INCONSISTENT WITH THE NTP DEA BIOASSAY AND FURTHER DEMONSTRATES THAT THERE IS INSUFFICIENT EVIDENCE TO JUSTIFY LISTING DEA UNDER NTP'S CRITERIA

The scientific literature, including publications by leading scientists at NIEHS, establishes the utility of the Tg.AC transgenic mouse model in determining the oncogenic potential of chemicals.[24] Based on this research, regulatory authorities, such as the Food and Drug Administration (FDA) and the International Conference on Harmonization of Technical

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21 As discussed in more detail below, ethanol has been demonstrated to increase the cellular demand for choline in rodents by increasing the oxidation of choline to betaine. Reference [3]; Reference [4]. This results in decreased choline levels. (*See* Stott Outline, ¶ 3.c.ii.) As discussed below, there is strong evidence that DEA has similar effects on choline levels.

22 *See* discussion below.

23 The Panel's calculations of dosages of ethanol administered to mice of varying body weights are provided in Table C, appended hereto. Animals were administered ethanol in doses as high as approximately 1,100-1,500 mg/kg/day. Similarly treated mice have been observed to begin grooming activities on the application site soon after dosing, Reference [5], resulting in the ingestion of at least a portion of the applied ethanol. Evidence that the amount of ethanol ingested likely was significant is discussed in more detail below.

24 *See, e.g.*, Spalding, *et al.* (1999). "Development of a transgenic mouse model for carcinogenesis bioassays: Evaluation of chemically induced skin tumors in Tg.AC mice." *Toxicol. Sci.* 49:241-254; Tennant, *et al.* (1995). "Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models." *Environ. Health Perspect.* 103:942-950; Eastin, *et al.* (1998). "The National Toxicology Program evaluation of transgenic mice as predictive models for identifying carcinogens." *Toxicol. Pathol.* 26:461-473.

Requirements for Registration of Pharmaceuticals for Human Use (ICH), have recently accepted the Tg.AC model as a mouse carcinogenicity study that may be used in lieu of the standard two-year mouse bioassay.[25] Accordingly, results from a properly conducted Tg.AC transgenic mouse study should be considered a significant factor in evaluating the weight of evidence.

In reviewing the results of the NTP DEA two-year mouse bioassay, the NTP Technical Report does not even mention, let alone fully consider, the negative results of the NIEHS Tg.AC mouse study on DEA.[26] DEA was administered in the transgenic mouse study at dose levels that significantly exceeded the highest dose used in the NTP bioassay.[27] Given the predictive value of the Tg.AC transgenic mouse model, the NIEHS study is inconsistent with the NTP DEA two-year mouse bioassay. Moreover, as stated by the authors of the NIEHS study, the "absence of an effect in the transgenic animals . . . is not necessarily representative of a false-negative result, but rather an indication that the conventional bioassay has given a false positive result."[28] The negative Tg.AC mouse study on DEA therefore further shows that the single positive NTP mouse bioassay on DEA does not constitute sufficient evidence that DEA is "reasonably anticipated to be a human carcinogen," and DEA thus should not be listed in the *RoC*. This is particularly the case given the other confounding and limiting factors discussed

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25 *Guidance for Industry, S1B Testing for Carcinogenicity of Pharmaceuticals* (July 1997) at 3 and 8, U.S. Department of Health and Human Services, Food and Drug Administration, and International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

26 Reference [2].

27 *Id.* at 218.

28 *Id.* at 221.

below that undermine the validity of the NTP DEA two-year mouse bioassay, such as use of the ethanol vehicle and the $B6C3F_1$ mouse's very high spontaneous liver tumor rate and genetic predisposition to develop liver tumors.

VII. THE NTP MOUSE STUDY SHOULD NOT BE USED AS A BASIS FOR LISTING DEA IN THE *RoC* BECAUSE OF TECHNICAL LIMITATIONS WHICH PRECLUDE IT FROM CONSTITUTING SUFFICIENT EVIDENCE THAT DEA IS "REASONABLY ANTICIPATED TO BE A HUMAN CARCINOGEN"

A. The Obesity of the Mice Was a Significant Confounding Factor in the NTP Bioassay

The body weights of the mice used in the study, particularly among the females, were unusually high.[29] The average maximum weights reached during the study were approximately 55 g and 52 g among the female and male groups, respectively.[30] During the 1980's, the average maximum weight for the mice at NTP for both male and female mice was in the range of 30-35 g.[31] Elevated body weight has previously been identified as a risk factor for liver tumor formation in this type of mouse.[32] Indeed, recent analyses and studies of the relationship between body weights and the incidence of liver tumors in the $B6C3F_1$ mouse, not

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29 MacGregor, J.A. and Bell, T. (1997). "NTP: The Chronic Toxicity/Carcinogenicity Dermal Studies of Diethanolamine in Fischer 344 Rats and B6C3F1 Mice -- Audit and Technical Study Review" at 4.

30 Reference [1] at 40-41.

31 Letter from Ernest E. McConnell to James G. Keay, Ph.D., Reilly Industries, Inc. (May 28, 2001) at 2 (McConnell Letter) (Attachment 3).

32 Seilkop, S.K. (1995). "The Effect of Body Weight on Tumor Incidence and Carcinogenicity Testing in $B6C3F_1$ Mice and F344 Rats." *Fund. Appl. Toxicol.* 24:247-259.

considered by NTP, reviewed in the context of an NTP mouse bioassay on pyridine, indicate that the obesity of the mice in the NTP chronic mouse bioassay was a critical confounding factor that makes the study insufficient as evidence of carcinogenicity. These recent studies and analyses indicate that $B6C3F_1$ mice are in effect programmed to develop liver tumors when they become obese. As a consequence, the biological significance of a finding of increased liver tumors in obese $B6C3F_1$ mice is highly questionable. This information is discussed in letters from Ernest F. McConnell and Judith A. MacGregor,[33] appended hereto as Attachments 3 and 4, which address the issue of obese mice in the context of the NTP bioassay on pyridine.

B. Data Concerning the Effect of Ethanol on Choline Levels, and Other Evidence of Ethanol's Effects As a Promoter, Not Considered in the NTP Technical Report, Indicate That Ethanol Was a Significant Confounding Factor in the NTP Bioassay

A significant body of data not considered by NTP in the Technical Report establishes that the use of ethanol as the vehicle in the NTP DEA bioassay was a significant confounding factor and therefore that the bioassay cannot be used, consistent with sound scientific principles, to justify listing DEA as a carcinogen in the *RoC*.

Despite the fact that DEA is water soluble, ethanol was chosen as the vehicle by which DEA was administered to the mice. Moreover, the application site was unoccluded and available for grooming. The role of ethanol in the absorption or toxicity/carcinogenicity of DEA in the NTP study is unknown, although repeated ethanol administration likely altered the skin

[33] Letter from Judith A. MacGregor, Ph.D., D.A.B.T., to Dr. George Alexeeff, OEHHA (May 31, 2001) at Item No. 1; McConnell Letter at 1-4.

barrier by denaturing/solubilizing epidermal proteins and lipids and/or enhanced ingestion of DEA by stimulating more active grooming. The dosage level of the ethanol used was relatively high.[34]

More importantly, ethanol has been demonstrated to be a liver cancer promoter or modifier in studies on rodents indicating that the administration of ethanol enhances the incidences of liver carcinomas and/or other liver tumors induced by vinyl chloride and N-nitrosodiethlylamine (NDEA).[35] It is widely regarded as a carcinogenic risk factor, including for liver cancer.[36]

Moreover, a significant body of data indicates that ethanol causes the loss of, and increases the demand for, choline in liver cells of rodents. These effects exacerbate the mechanism by which DEA is believed to potentially induce tumors in mice, *i.e.*, causing choline

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34 Animals were administered ethanol vehicle in doses as high as approximately 1,100-1,500 mg/kg/day, as discussed above. (*See* Table C, appended hereto.)

35 *See, e.g.*, Radike, *et al.* (1981). "Effect of ethanol on vinyl chloride carcinogenesis." *Environ. Health Perspect.* 41:59-62 (vinyl chloride); Driver and McLean (1986). "Dose-response relationships for initiation of rat liver tumours by diethylnitrosamine and promotion by phenobarbitone or alcohol." *Food Chem. Toxicol.* 24:241-245 (NDEA); Takada, *et al.* (1986). "Effects of ethanol on experimental hepatocarcinogenesis." *Hepatology* 6:65-72 (NDEA); IARC (1988). *Monographs on the Evaluation of Carcinogenic Risks to Humans, Alcohol Drinking*, Vol. 44 at 108, 114, and 252. While the IARC Working Group noted the small number of animals used in the Driver and McLean study, IARC did not indicate that that study was invalid. *See* IARC *Monograph*, Vol. 44 at 108.

36 IARC *Monograph*, Vol. 44; NTP, *Draft RoC Background Document for Alcoholic Beverage Consumption (Dec. 2-3, 1998)* (1998), at 1, 1-1 (proposing that alcoholic beverages are "known to be carcinogenic to humans" and noting that ethanol and water are the main constituents of most alcoholic beverages).

deficiency.[37] Evidence of the choline depleting effects of ethanol in rodents is provided by the following findings:[38]

- Ethanol increases the rate of choline uptake (demand) by the liver of rats.[39] (*See* Stott Outline, Table D.) Increased choline demand (uptake) equates to increased oxidation (loss).

- As noted above, ethanol has been demonstrated to increase the cellular demand for choline in rats by increasing the oxidation of choline to betaine.[40] Ethanol appears to deplete choline via increased betaine oxidase metabolism of choline as a result of increased demand for methionine formation.[41] (*See* Stott Outline, Figure B.)

The importance of ethanol's effects on choline metabolism with regard to the NTP DEA mouse study is underscored by the fact that the DEA-ethanol mixture was dermally applied without implementing measures to restrict access to the application site. Failure to restrict access in turn allowed grooming and subsequent ingestion of both the ethanol vehicle and DEA. Based on the following data, it is likely that the amount of both ethanol and DEA ingested was significant:

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37 *See* discussion below regarding DEA's mode of action.

38 While the research on the effects of ethanol on the loss and increased demand for choline in liver cells has been conducted on rats, the effects in mice and rats should be similar. Rats and mice both have high choline oxidase activity. Reference [6]. Animals having high choline oxidase activity are likely "much more susceptible to possible ethanol-induced lipotropic [*e.g.*, choline] deficiency than others." Reference [7].

39 Reference [3]; Reference [7]; Reference [8].

40 As discussed below, DEA has similar effects on choline metabolism.

41 Reference [4].

- Grooming activity has been reported immediately following application of an ethanol-DEA solution to the skin of a mouse.[42]

- Blood levels of DEA in mice administered DEA dermally with access to the application site are approximately 35% higher than in mice prevented access to the site, indicating that some ingestion was occurring.[43]

- The small amount of water in the 95% ethanol solution used in the NTP DEA study would have retarded evaporation of ethanol from the skin of a mouse.

- As discussed above and shown in Table C (appended hereto), a relatively high dosage of ethanol was delivered to the mice in the NTP DEA study.

In sum, available data indicate that use of ethanol in the NTP DEA study was a significant confounding factor. Accordingly, the study cannot constitute sufficient evidence to justify the listing of DEA under the NTP criteria.

C. High Dose Effects in Treated Mice in the NTP Bioassay Suggest That the Experimental Animals May Have Been Administered DEA Doses in Excess of the Maximum Tolerated Dose, Thus Further Compromising the Study's Results

The survival of high and intermediate group mice was significantly depressed relative to controls. This suggests that doses in the NTP study may have exceeded the maximum tolerated dose. Moreover, DEA was toxic to mice that received 80 and 160 mg/kg by topical application for 13 weeks.[44] Despite this toxicity, NTP selected 160 mg/kg as the high dose for

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42 Reference [5].

43 *Id.*

44 Reference [1] at 49.

the two-year mouse study, and 80 mg/kg as the mid dose. According to NTP, the most significant responses in the 13-week study occurred in the liver, kidney, and skin. Liver weights were significantly increased in all dosed groups of females and in males treated with 160 mg/kg or greater. Associated with the increase in liver weights was an increase in the incidence and severity of cytological alteration of hepatocytes. Kidney weights were significantly increased in all dosed groups of males and females. Similarly, acanthosis was present at the site of application in all animals treated with DEA.[45]

These toxic effects, together with the overall depression of survival rates in the high and intermediate groups in the two-year study, show that the doses used in the NTP study may have exceeded the maximum tolerated dose, particularly for female mice.

D. The Validity of the NTP Mouse Bioassay As a Dermal Study Was Compromised by the Likely Significant Chronic Ingestion of DEA During the Study

Although the study was designed as a skin-painting study, the test site was non-occluded (uncovered), leaving ample opportunity for chronic ingestion of a significant amount of DEA and ethanol during grooming. As noted above, blood levels of DEA in mice administered DEA dermally with access to the application site are approximately 35% higher than in mice prevented access to the site, proof that some ingestion was occurring. Human exposures to DEA are normally dermal, however, and do not typically occur through oral ingestion.

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45 *Id.*

VIII. NTP SHOULD GIVE FULL WEIGHT TO DATA SHOWING THAT MOUSE LIVER TUMORS ARE NOT RELEVANT TO CARCINOGENICITY HAZARD IDENTIFICATION AND DISCOUNT THE NTP BIOASSAY ON THOSE GROUNDS ALONE

In the Technical Report, NTP did not consider data regarding the relevance of mouse liver tumors for human risk assessment.[46] Indeed, EPA has recognized that mouse liver tumors as being "of questionable significance regarding human risk assessment."[47]

Liver tumors in the $B6C3F_1$ mouse are particularly suspect.[48] The $B6C3F_1$ mouse has a high background incidence of spontaneous liver tumors, which can vary several fold with different stocks of animals and from study to study.[49] Recent studies have demonstrated a strong correlation between body weight and the development of liver tumors in the $B6C3F_1$ mouse.[50] Scientists at NIEHS have concluded that "liver tumor incidence in $B6C3F_1$ mice is strongly correlated with body weight."[51] Much of the variance in liver tumor incidence in chronic mouse

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46 *See, e.g.,* Carmichael, N.G., Enzmann, H., Pate, I., and Waechter, F. (1997). "The Significance of Mouse Liver Tumor Formation for Carcinogenic Risk Assessment: Results and Conclusions from a Survey of Ten Years of Testing by the Agrochemical Industry." *Environ. Health Perspect.* 105:1196-1203, at 1196.

47 EPA, Risk Assessment Forum, *Report on the Workshop on Cancer Risk Assessment Guidelines Issues* (Nov. 1994) at 4-21.

48 *See, e.g.,* International Life Sciences Institute (ILSI), *Fifth Workshop on Mouse Liver Tumors, Summary Report* (Nov. 7-9, 1994) at 45 (Dr. Robert Maronpot of NIEHS questioned the appropriateness of the mouse liver tumor response as an index of potential human risk).

49 *See, e.g.,* Carmichael, *et al.* (1997) at 1198; ILSI Workshop at 7, 16, 32, 42.

50 ILSI Workshop at 61.

51 *Id.* at 3; *see also id.* at 45, 60-61.

studies is now attributed to body weight differences.[52] As pointed out by Drs. Leakey and Turturro at the National Center for Toxicological Research, "[t]his creates problems for interpretation of chronic cancer bioassays that use this mouse strain."[53]

Further, the causative factors for human liver cancer and mouse liver tumors are quite distinct. Human liver tumors, rare in developed countries, are associated with alcohol-induced liver cirrhosis, hepatitis, and dietary aflatoxin.[54] In human liver cancer, cirrhosis of the liver precedes the development of cancer in over 80 percent of the instances, whereas cirrhosis rarely occurs in mice in association with liver tumors.[55] Recent studies have demonstrated that liver-tumor sensitive strains of mice develop liver tumors by a mechanism that does not have a counterpart in humans. In particular, in spontaneous mouse liver tumors in the $B6C3F_1$ mouse, a high incidence of mutations has been observed in the *ras* family of proto-oncogenes.[56] Such mutations are rarely found in human liver cancers.[57] The $B6C3F_1$ mouse also is defective in its ability to maintain normal methylation of DNA,[58] which is critical to control gene expression.[59]

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52 *Id.* at 45, 60.

53 *Id.* at 61.

54 *Id.* at 33, 35, 37-39, 42; Carmichael, *et al.* (1997) at 1202.

55 *Id.* at 39, 46.

56 *Id.* at 22, 36-37.

57 *Id.* at 36-37.

58 DNA methylation refers to the methylation of cytosine to 5-methylcytosine. *See id.* at 15.

59 *Id.* at 13-16, 42. Hypomethylation is believed to contribute to carcinogenesis by a secondary threshold mechanism. *Id.* at 16.

Mouse liver tumors caused by non-genotoxic compounds appear to operate by high dose secondary mechanisms that do not come into play at the low doses of human exposure.[60] Many compounds that induce mouse liver tumors have been found to stimulate liver cell proliferation, either by directly stimulating liver cell proliferation or by indirectly causing proliferation as part of the compensatory regenerative response to cell toxicity. Liver enzyme induction is also seen with some compounds that induce mouse liver tumors. The induction of liver enzymes is viewed as a reversible response of the liver to an excessive body load. For all these reasons, mouse liver tumors, particularly in $B6C3F_1$ mice, generally are high dose phenomena unique to the test species and, therefore, not relevant for human hazard identification. The Technical Report, however, does not consider these important data in reaching its conclusion.

IX. THE NTP MOUSE BIOASSAY DOES NOT SUPPORT LISTING UNDER NTP'S CRITERIA BECAUSE MECHANISTIC RESEARCH SPECIFICALLY ON DEA INDICATES THAT, TO THE EXTENT DEA CAN POTENTIALLY INDUCE TUMORS IN MICE, IT DOES SO BY A MECHANISM THAT IS NOT RELEVANT TO HUMANS

A number of studies have been conducted to elucidate the mechanism by which DEA potentially may induce tumors in mice. This research provides convincing evidence of the likely operative mechanism specifically for DEA, and also indicates that DEA does not pose a risk of cancer to humans.

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60 *Id.*

Current research indicates that to the extent DEA can induce tumors in mice, it does so by causing chronic choline deficiency. The salient aspects of this research may be summarized as follows:

- DEA has been shown to cause decreases in intracellular choline pools in cultured mammalian cells and in rodents [References 5, 9, 10]. *In vitro*, DEA has been shown to competitively inhibit choline uptake in treated Chinese Hamster Ovary cells and Syrian Hamster Embryo primary cultures [Reference 9]. *In vivo*, DEA has been shown to cause up to an 85% depression in the primary choline pool, phosphocholine, in mice, when administered at the high dose used in the NTP study. [Reference 5]. (*See* Stott Outline, Figure C.)

- Chronic choline deficiency has been linked to liver tumor formation in rats and mice in a number of studies [References 11-19]. The mechanism appears to involve a shift in second messenger stimulated chronic activation of protein kinase C isoforms (PKC) with subsequent chronic elevation in hepatocellular and renal cell S-phase DNA synthesis [References 19-23], which is associated with increased cell turnover.[61] Elevation in S-phase DNA synthesis is well accepted as a common characteristic of nongenotoxic carcinogens, including choline deficiency [References 24, 25]. Hypomethylation of DNA has also been associated with longer-term choline deficiency [References 26, 27]. Indeed, levels of the important endogenous methylating agent S-adenosylmethionine were observed to be depressed in mice administered the high DEA dose in the NTP study. [References 28, 29]. (*See* Stott Outline, Figure D.) Direct evidence of the involvement of choline deficiency in DEA-induced tumorigenic activity has come from an *in vitro* cell transformation assay which reportedly can detect nongenotoxic carcinogens [Reference 30]. Lehman-McKeeman and Gamsky [Reference 10] reported significant choline deficiency in Syrian Hamster embryo cells exposed to DEA *in vitro*. Supplementation of the media with choline inhibited DEA-induced morphological transformation of the cells (*i.e.*, resulted in a negative test response). Thus, in absence of choline supplementation DEA caused choline deficiency and increase in cell transformation. With choline supplementation, there was no such increase in cell transformation.

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61 Consistent with this mechanism, chronic increases in hepatocellular S-phase DNA synthesis have been observed in the liver of $B6C3F_1$ mice administered potentially tumorigenic dosages of DEA. (*See* Stott Outline, Figure A and reference.)

- Additional recent research further supports the conclusion that any tumorigenic effect of DEA administered in ethanol vehicle is due to choline deficiency. That research has demonstrated increased S-phase DNA synthesis in liver and kidney cortex and outer medulla of mice administered DEA via skin painting, as in the NTP bioassay. Specifically, zonal specific increases in S-phase synthesis were found in liver and kidney of mice administered the high dosage of DEA in the NTP mouse bioassay in an ethanol vehicle. (Further, increased apoptosis was found in mouse liver after 13 weeks of application at that dosage.[62]

- The NTP Technical Report suggests that DEA tumorigenesis may be related to the metabolic incorporation of DEA into phospholipids.[63] Data accumulated to date, however, do not support this hypothesis:

  - There has been evidence of incorporation of DEA altered phospholipids in rats at the dosages tested in the NTP bioassay, yet no tumors were noted in these animals. Evidence of DEA altered phospholipids in rats at those dosages is provided by the observation of microcytic anemia in male and female F344/N rats administered 63 and 32 mg/kg/day DEA, respectively, via their drinking water for 13 weeks [Reference 33], based upon the following considerations. Metabolites of DEA have been shown to accumulate in red blood cells (RBCs) (erythrocytes) of rats administered ^{14}C-DEA [Reference 34]. Erythrocytes are known to undergo changes in cell shape upon alterations in membrane phospholipids [References 35, 36]. These changes could logically be expected to decrease the functional half-life of RBCs, resulting in the onset of microcytic anemia.

  - The regional location of increased S-phase DNA synthesis, indicative of increased cell turnover, in the liver of mice administered DEA over a 3-7 day period is not consistent with the normal location of hepatocyte regeneration in the lobule. New synthesis of hepatocytes occurs primarily in the periportal regions of the hepatic lobule. It follows that the highest level of DEA-altered membrane phospholipids following a short DEA dosing period would also be located

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62 Mellert, W., Gernbardt, C., and Hildebrand, B. (2000). "Diethanolamine -- S-Phase Response Study in Liver and Kidney of Male B6C3F$_1$ Mice, Dermal Administration for 1, 4 and 13 Weeks." BASF Project No.: 99C0299/99041.

63 Phospholipids are structural components of cell membranes [References 31, 32].

> primarily in the periportal regions of the hepatic lobule. [Reference 37]. Therefore, if altered membrane lipids had been responsible for an increase in hepatocellular turnover, such an increase in turnover would be expected to have been either pan-lobular or primarily periportal. To the contrary, most hepatic S-phase synthesis following administration of 160 mg/kg/day DEA occurred in hepatocytes bordering the central vein [Reference 23]. Accordingly, it is apparent that DEA does not increase cell turnover as a result of alteration of membrane phospholipids.

IARC has concurred that any potential tumorigenic effect of DEA in mice is likely due to choline deficiency, concluding that a "[d]iethanolamine-induced choline deficiency thus provides a mechanism for the tumorigenesis noted in mice but not in rats."[64]

The results of numerous studies indicate that humans are resistant to the development of choline deficiency relative to rodents. Because choline deficiency has been identified as the likely mode of potential tumorigenic action of DEA in mice, reasonably

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64 IARC *Monograph*, Vol. 77 at 372. The *Monograph* states, in further detail:

> In mice, diethanolamine alters choline homeostasis in a manner resembling choline deficiency. Stott *et al.* (2000) showed that diethanolamine induced choline deficiency and depleted several choline-containing compounds in $B6C3F_1$ mice, while Lehman-McKeeman & Gamsky (1999, 2000) found that diethanolamine inhibited the uptake of choline into mammalian cells.
>
> It is known that deprivation of choline in the diet of rodents predisposes to the appearance of hepatocellular carcinomas (Zeisel, 1996). Diethanolamine-induced choline deficiency thus provides a mechanism for the tumorigenesis noted in mice but not in rats.

Id. See also detailed description of mechanistic research in the IARC *Monograph* at 368.

anticipated exposures that might result from the normal production and use of DEA will not appear to pose a carcinogenic risk to humans. Significant findings to date include:

- Evidence of choline deficiency has been found in humans only under extreme conditions that preclude chronic situations necessary to pose a tumorigenic risk. Patients suffering malnutrition and liver cirrhosis and thus compromised ability to synthesize choline, or undergoing long-term total parenteral feeding were reported to display symptoms of choline deficiency consisting of altered liver function and/or hepatic steatosis [References 38-40]. These changes were readily reversible upon providing choline or lecithin supplementation. In contrast, prolonged fasting of healthy subjects resulted in only modest changes in plasma choline levels and no evidence of hepatic injury [Reference 41]. Choline levels rapidly rebounded following resumption of a normal diet.

- Nonhuman primates reportedly were much more resistant to development of choline deficiency related liver pathology than rats [References 42, 43].

- An enzymological basis for at least some of the resistance of higher species to development of choline deficiency has been identified. Choline undergoes oxidation via a well-established pathway to produce betaine which is instrumental in methionine synthesis. The rate at which this reaction occurs has been found to be much higher in rodents than in higher mammals, including humans [References 6, 44].

An additional important consideration is that exposure to humans from the production or use of DEA or products containing DEA would be primarily by the dermal route. Research indicates that DEA is absorbed dermally in humans at a very low rate compared to mice. Published data show that DEA skin penetration is much greater through mouse skin than it is through human skin.[65] In addition, work done by CTFA measuring DEA absorption from

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[65] Sun, J.D., *et al.* (1996). "In Vitro Skin Penetration of Monoethanolamine and Diethanolamine Using Excised Skin from Rats, Mice, Rabbits, and Humans." *J. Toxicol. Cut. & Ocular Toxicol.* 152(2):131-146.

model cosmetic formulations through human skin *in vitro* has shown very low absorption.[66] The low rate of dermal absorption of DEA in humans further establishes that any reasonably anticipated exposure to DEA would not induce choline deficiency in humans, and hence would not pose a human cancer risk.

In sum, from the research discussed above and the foregoing considerations, it is clear that any reasonably anticipated human exposures to DEA that could result from the normal production and/or use of DEA or products containing DEA would not cause choline deficiency in humans. Accordingly, DEA does not appear to pose a carcinogenic risk to humans and should not be listed.[67]

X. EXTENSIVE EVIDENCE SHOWS THAT DEA IS NOT GENOTOXIC, AND THERE IS NO OTHER AVAILABLE CORROBORATIVE EVIDENCE THAT WOULD SUPPORT A LISTING IN THE *RoC*

DEA has been extensively tested for potential genotoxicity in numerous tests and test systems and virtually uniformly has been found to be negative for mutagenicity. DEA has been thoroughly evaluated for mutagenic potential in a number of bacterial mutation assay

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66 A manuscript reporting the results of these studies is being prepared for publication.

67 While the Panel believes that the existing mechanistic research on DEA sufficiently demonstrates the mechanism by which DEA in an ethanol vehicle potentially may induce tumors in mice and that such mechanism is not relevant to human risk, the Panel is currently planning additional confirmatory research on these matters. The Panel would be pleased to share the nature of this additional research with NTP.

systems, which consistently have been negative.[68] In addition, DEA has been shown to be negative in the mouse lymphoma L5178Y mammalian cell mutagenicity assay.[69] DEA also failed to cause the transformation of another mammalian cell type, Chinese hamster embryo cells, to a more anaplastic state *in vitro*.[70] The addition of liver enzymes isolated from rats or hamsters treated with PCBs to these assays did not alter the negative responses obtained.

The potential of DEA to cause chromosomal damage has been extensively evaluated. Clastogenesis assays of DEA have been carried out *in vitro* in a number of test organisms ranging from yeast to cultured cells derived from ovary, lung, and liver tissues.[71] Results of these tests have been uniformly negative with or without the addition of metabolic fractions recovered from PCB-induced rat liver. DEA has also failed to demonstrate clastogenic activity *in vivo* in a mouse micronucleus test conducted using animals that had been administered

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68 Dean, B.J., *et al.* (1985). "Genetic toxicology testing of 41 industrial chemicals." *Mutat. Res.* 153:57-77; Haworth, S., *et al.* (1983). "*Salmonella* mutagenicity test results for 250 chemicals." *Environ. Mutagen* 5 (1): 3-142; Hedenstedt, A. (1978). "Mutagenicity screening of industrial chemicals: seven aliphatic amines and one amide tested in the *Salmonella*/microsomal assay (abstract)." *Mutat. Res.* 53:198-199.

69 Myhr, B.C., Bowers, L.R., and Caspary, W.J. (1986). "Results from testing of coded chemicals in the L5178Y TK+/- mouse lymphoma mutagenesis assay (abstract)." *Environ. Mutagen.* 7(3):58.

70 Inoue, K., Sunakawa, T., Okamoto, K., and Tanaka, Y. (1982). "Mutagenicity tests and *in vitro* transformation assays on triethanolamine." *Mutat. Res.* 101:305-313.

71 Dean, *et al.* (1985); Loveday, K.S., *et al.* (1989). "Chromosome aberration and sister chromatid exchanges in Chinese hamster ovary cels *in vitro*: II. Results with 20 chemicals." *Environ. Mol. Mutagen.* 13:60-94; Melnick, R.L. (1992). NTP, *NTP Technical Report on Toxicity Studies of Diethanolamine Administered Topically and in Drinking Water to F344/N Rats and $B_6C_3F_1$ Mice.* Publ. 92-3343. National Institutes of Health, Bethesda, MD.

up to 1,250 mg/kg/day via skin painting for 13 weeks.[72] While the NTP Technical Report mentions a single positive *in vitro* assay, it concludes that the data indicate "little evidence for [mutagenic] activity."[73]

Finally, DEA does not belong to a well-defined, structurally related class of substances whose members are listed in a previous *RoC* and there are no other corroborative data that would justify a listing.

XI. THE NTP DEA CONDENSATE STUDIES MAY NOT BE RELIED UPON IN EVALUATING THE CARCINOGENICITY OF DEA AND, IN ANY EVENT, DO NOT SUPPORT THE CONCLUSION THAT DEA MAY "REASONABLY BE ANTICIPATED TO BE A HUMAN CARCINOGEN"

There are three NTP studies on DEA condensates -- the cocamide condensate study,[74] the lauramide condensate study,[75] and the oleamide condensate study.[76] The oleamide condensate study reported no increase in tumors. The lauramide condensate study reported an increase only in liver tumors in mice. Only the cocamide condensate study reported an increase in tumors in both liver neoplasms and renal tubule neoplasms (male mice only). The NTP Technical Report on the cocamide study states that these increases were "associated" with the

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72 Melnick (1992).

73 Reference [1] at 22.

74 Reference [45].

75 Reference [46].

76 Reference [47].

concentration of free DEA present as a contaminant in the DEA condensate.[77]

Neither the NTP cocamide study nor the lauramide study can be used to support the conclusion that DEA itself is a carcinogen, and the cocamide study cannot be used to support the conclusion that DEA induces a combination of malignant and benign tumors at multiple tissue sites.

As concluded by the IARC DEA Working Group in its recent classification of DEA as a Group 3 chemical,[78] and as outlined in Table A (appended hereto), numerous factors establish that the DEA condensate studies cannot be used for evaluating the carcinogenicity of DEA *per se* and therefore, that those studies, by definition, do not represent independent confirmation of the DEA bioassay findings. These factors include, but are not limited to, the following:

- The DEA condensate studies were bioassays of complex mixtures of imprecise composition of many chemicals, of which DEA comprised only a small proportion. Therefore, the condensate studies "were not designed as, and did not represent, conventional or adequate carcinogenesis bioassays of [DEA]" and "cannot be used for this purpose."[79]
- The condensates contained relatively high levels of unknown organic impurities.

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77 Reference [45] at 55.

78 *See* IARC *Monograph*, Vol. 77 at 362. (Page attached as Attachment 5.)

79 *Id.*; *see also* Hard Letter at 3.

- There is substantial uncertainty as to the concentrations of free DEA that actually were present in the condensates. Accordingly, the actual dosage of free DEA administered in the condensate studies is unknown.

- There is not a consistent pattern of toxicity in *in vitro* and *in vivo* studies among the different DEA condensates and DEA itself, indicating that the condensates have a far more complex mode of action than could be attributed to DEA itself.

- The fact that the condensate amides were complex mixtures could have influenced DEA absorption kinetics and toxicity.

- Nitrosamine quantitation by high-performance liquid chromatography (HPLC) detected the known genotoxic carcinogen, N-nitrosodiethanolamine, at significant concentrations in the cocamide and lauramide condensates.[80]

More specifically, the condensates were complex mixtures, of which DEA comprised at most only a small proportion. "Unknown organic impurities" and a variety of amides, other than the nominal amide, comprised a majority of the cocamide and oleamide test materials and a substantial portion of the lauramide test material. Given these facts, it is not possible to ascertain whether responses observed in the condensate studies were due to individual components, a subset of components, or the whole mixture. The NTP bioassays, therefore, simply were not designed to make this evaluation, clearly were not designed to evaluate the oncogenicity of DEA *per se*, and cannot be used for that purpose.

Moreover, the levels of DEA in the condensate test materials were not determined analytically, but instead represent estimated values based upon information supplied by the

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[80] N-nitrosodiethanolamine was detected at concentrations of 219 ppb and 3,600 ppb in the cocamide and lauramide condensates, respectively. Hard Letter at 3.

manufacturer [References 1, 45-47].[81] This, together with the fact that the lauramide, cocamide, and oleamide NTP reports each specify differing values for the percent of DEA content at different points either within the same report, or in the case of the oleamide study, between the draft and final reports, make highly uncertain the "dose" of free DEA that was administered in the condensate bioassays.[82] Evaluation of dose-response with respect to DEA is thus inappropriate on those grounds alone.

Further, as in any mixture, components may influence the dermal absorption of any single component of that mixture. Accordingly, the kinetics of absorption of any free DEA in a condensate mixture may be altered by condensate components, thereby affecting the systemic dosages of DEA administered and further compounding the uncertainties in the interpretation of the condensate studies.

Finally, inconsistencies in the spectrum of *in vitro* and *in vivo* genotoxicity assay responses and responses in a short-term oncogenicity bioassay, suggest a more complex mode of action of the condensates than can be attributed simply to potential DEA-induced oncogenic activity. Lauramide was positive in a short-term transgenic mouse model capable of identifying

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81 *See also* National Toxicology Program (1997). *Toxicology and Carcinogenesis Studies of Coconut Oil Acid Diethanolamine Condensate (CAS No. 68603-42-9) in F344/N Rats and B6C3F$_1$ Mice (Dermal Studies)*. NTP TR 479, NIH Publication No. 97-3969 (Draft).

82 *See, e.g.*, Reference [45] at 15 and 52, indicating concentrations of DEA in the cocamide condensate ranging from 4%-8.5% in one place, and an estimated concentration of 18.2% in another; Reference [46] at 17 and 48, indicating concentrations of DEA in the lauramide condensate of 5% and 0.83%, respectively. *See* Table A, appended hereto, for discrepancies in the specified DEA concentration for all the condensates. S*ee also* Hard Letter at 3.

tumor promotors [Reference 2] and an *in vitro* measure of chromosomal effects in Chinese Hamster Ovary cells [Reference 46]. Both DEA and cocamide were negative in these assays [References 1, 2, and 45]. The cocamide mixture, however, was positive in a mouse micronucleus test while DEA was negative, even at a much higher dosage -- *i.e.*, positive results for cocamide at 800 mg/kg/day compared to negative results for DEA at 1,200 mg/kg/day [References 1, 45]. These factors preclude drawing firm conclusions regarding the role of free DEA in the response of mice to chronic administration of the condensate test materials.[83]

Accordingly, the condensate studies cannot be considered a study on DEA itself.

Moreover, these studies also suffered from many of the same deficiencies of the NTP DEA mouse bioassay discussed above. In addition, the number of renal tubule adenomas observed in the high dose in the cocamide study was only two, the same number observed in the controls of the DEA study, and even if the nominal percentage of DEA stated to be present in the cocamide test material were accurate, the amount of DEA present in the high dose group would be roughly the same as in the low dose group in the DEA study. Thus, the results would be inconsistent with those of the NTP DEA bioassay where, as noted above, the numbers of renal carcinomas reported at the low and mid doses were one and zero, respectively.

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[83] The spectrum of *in vitro* and *in vivo* genotoxicity assay and transgenic oncogenicity responses are set forth in detail in Table A, appended hereto. The inconsistencies are also shown in a more schematic format in Table B, appended hereto.

XII. NTP SHOULD FOLLOW THE LEAD OF IARC AND CONCLUDE THAT DEA IS NOT CLASSIFIABLE AS TO ITS CARCINOGENICITY TO HUMANS AND HENCE MAY NOT REASONABLY BE ANTICIPATED TO BE A HUMAN CARCINOGEN UNDER THE NTP CRITERIA

After evaluating the NTP DEA bioassay on mice and rats, the negative Tg.AC transgenic mouse bioassay, the genotoxicity data, which IARC concluded did not indicate DEA is genotoxic, and other relevant information, IARC found that DEA is "not classifiable as to its carcinogenicity to humans" and designated it as a Group 3 chemical under IARC's classification system.[84] The Panel believes that IARC based its conclusion on sound scientific grounds and therefore that NTP should similarly conclude that DEA may not reasonably be anticipated to be a human carcinogen and should not be listed.

CONCLUSION

For the reasons discussed above, the Panel believes that the available studies and data do not establish that DEA is "reasonably anticipated to be a human carcinogen" and therefore that NTP should determine that listing of DEA in the *RoC* would not be appropriate.

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[84] IARC *Monograph*, Vol. 77 at 372-374.

TABLE A. Condensate Amides Composition and Selected Toxicity Data.*

(mkd = Mg/Kg/Day)

STUDY	% DEA Reported	Test Material Purity (%)	% "Unknown Organic Impurities"	NDELA (ppb)	Significant Toxicity Data
Oleamide Study [TR 481]	0.19 ["based on the amine value supplied by the manufacturer"]**	48	22 [another 30% as unknown fatty acid amides]	68	B6C3F1 (M&F): No increased incidence of liver tumors at ≤30 mkd. Tg.AC Transgenic Mouse Model: Negative at approx. 40 mkd.
Lauramide Study [TR 480]	0.83 & 5 [both values appear based upon manufacturers information]	90	9.2	3600	B6C3F1 (F): Numerical increased incidence of liver tumors at ≥100 mkd. No dose-response. CHO SCE Assay: Positive. Mouse MN Test: Negative at 800 mkd. Tg.AC Transgenic Mouse Model: Positive at approx. 400 mkd.
Cocamide Study [TR 479]	4-8.5 & 19.6 [latter value "based on calculations using the amine value supplied by the manufacturer"]	Identified 40% as Lauramide, Remainder mixed amides.	Not Reported	218	B6C3F1 (M&F): Increased incidence of liver tumors at ≥100 mkd. B6C3F1 (M): Increased incidence of kidney tumors at 200 mkd. CHO SCE Assay: Negative. Mouse MN Test: Positive at 800 mkd. Tg.AC Transgenic Mouse Model: Negative at approx. 250 mkd.
DEA Bioassay [TR 478]	>99% [analysis]			<LOD of 1000 ppb	B6C3F1 (M&F): Increased incidence of liver tumors at ≥40 mkd. B6C3F1 (M): Increased incidence of kidney tumors at ≥40 mkd. CHO SCE Assay: Negative. Mouse MN Test: Negative at 1250 mkd. Tg.AC Transgenic Mouse Model: Negative at approx. 800 mkd.

* References [1, 2, 45-47].

** Reference [47]. The draft cocamide study (at page 67) on the other hand reports 7.3% free DEA in the oleamide condensate based on "[d]ata provided by the manufacturer." *See also* Hard Letter.

Table B -- Schematic Comparison of Reported Results from NTP Mouse Bioassay and Other Key Toxicity Studies on DEA and DEA Condensates[85]

Study and Reported Results

Test Substance	NTP Bioassay ($B6C3F_1$ Mice)	Tg.AC Transgenic Mouse Study	Mouse Micronucleus Test	CHO SCE Assay
DEA	+ (M&F)	-	-	-
Cocamide Condensates	+ (M&F)	-	+	-
Lauramide Condensates	+ (F)	+	-	+
Oleamide Condensates	- (M&F)	-	nd	nd

nd = no data (not conducted)

85 References [1, 2, 45-47].

Table C. Calculated dosages of ethanol vehicle delivered mice of varying body weights (Equation: Dose (mg/kg/day) = (volume delivered x density (0.816) / Body Wt.).

	Body Weight (Kg)			
	0.03	0.04	0.05	0.06
Males				
Volume/Mouse (uL)				
Min. 41	1115.2	836.4	669.1	557.6
Max. 93	2529.6	1897.2	1517.8	1264.8
Females				
Volume/Mouse (uL)				
Min. 34	924.8	693.6	554.9	462.4
Max. 91	2475.2	1856.4	1485.1	1237.6

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Tab 1

September 14, 2000

Jonathon T. Busch
Manager, Alkanolamines Panel
Director, CHEMSTAR Panels
American Chemistry Council
1300 Wilson Boulevard
Arlington, VA 22209

ACC Reference No. ALK-LETTER-HARD

Dear Mr. Busch:

In relation to the risk assessment of diethanolamine, I wish to provide information on the issues listed below, representing my professional opinion based on 35 years of experience in toxicologic and experimental pathology. In addition, I was an active member of the IARC Working Group for the Evaluation of Carcinogenic Risks to Humans – Volume 77, Some Industrial Chemicals, during which session diethanolamine (DEA) was evaluated. Specifically, my role in that working group was Chairman of the Subgroup on Experimental Carcinogenicity Data.

Sequence of liver tumor development

Based on numerous experimental studies in both mice and rats, it has long been accepted by pathologists with an intimate working knowledge of rodent liver carcinogenesis that liver cancer develops according to a morphologic sequence of lesions. In the continuum, adenomas and carcinomas are sequential stages, with adenomas preceding and being able to progress into carcinomas (Ward et al, 1980; Harada et al, 1999). The proof of this lies in the facts that most small carcinomas can be observed to arise within an adenoma, and that carcinomas arising *de novo* or *in situ* rarely, if ever, occur (Goodman et al., 1991). Hepatoblastomas can be regarded as one extreme of this continuum because they almost exclusively occur within existing carcinomas. They are, therefore, considered to represent further progression to a more primitive or undifferentiated variant of hepatocellular neoplasm, and do not constitute a separate entity (NTP, 1997a; Harada et. al., 1999).

NTP carcinogenicity bioassay of DEA

In NTP's 2-year carcinogenicity (dermal application) assay of DEA (NTP, 1997a), hepatocellular tumor incidences in male and female mice at the 0, 40, 80 and 160 mg/kg dose-levels were as follows:

Males, adenoma 31/50 (62%), 42/50 (84%), 49/50 (98%), 45/50 (90%)
Males, carcinoma 12/50 (24%), 17/50 (34%), 33/50 (66%), 34/50 (68%)
Males, combined 39/50 (78%), 47/50 (94%), 50/50 (100%), 49/50 (98%)

Females, adenoma 32/50 (64%), 50/50 (100%), 48/50(96%), 48/50 (96%)
Females, carcinoma 5/50 (10%), 19/50 (38%), 38/50 (76%), 42/50 (84%)
Females, combined 33/50 (66%), 50/50 (100%), 50/50 (100%), 50/50 (100%)

At the IARC meeting, this increase in liver tumors was considered to be only *limited* evidence of carcinogenicity for several reasons. Firstly, the $B6C3F_1$ mouse is noteworthy for its very high spontaneous liver tumor incidence (Maronpot et. al., 1987). Accordingly, $B6C3F_1$ mice are also more susceptible to increased development of liver tumors after exposure to chemicals than are mice of other strains (Rao et al., 1988). High spontaneous incidence means that many liver cells are initiated inherently on the pathway to cancer, and a chemical compound can then enhance the clonal expansion of these initiated cells, enhancing in turn, further along the continuum, the sequential conversion of adenomas to a higher grade of neoplastic lesion, i.e., to carcinoma – a mechanism which explains the compound's apparent carcinogenicity. In point of fact, because of the high predilection of the $B6C3F_1$ mouse to liver tumor development, the assay can be viewed as not discriminating between promoters and true carcinogens. Furthermore, the vehicle control incidence, i.e. the spontaneous background, was very high in the NTP DEA study, being 78% for the combined incidence of adenomas and carcinomas in the male mice, and 66% in the females. The female control incidence, in fact, exceeded the historical control range cited by NTP for 2-year dermal studies in female $B6C3F_1$ mice (NTP, 1997a), an additional factor which detracts from the significance of the bioassay results. There is a growing body of opinion that views the $B6C3F_1$ bioassay as inappropriate for predicting or assessing cancer risk in humans (Grisham, 1996; Counts et. al., 1996). One persuasive reason for this is that the $B6C3F_1$ mouse appears to have a diminished capacity to maintain normal methylation status when exposed to tumor promoting stimuli (Counts et. al., 1996). Thus, global hypomethylation occurs in this strain, resulting in an increased sensitivity to liver carcinogenesis. In contrast, human cells are capable of maintaining a more stable methylation status (Counts et. al., 1997; Dragan et al., 1998).

Carcinogenicity bioassays of DEA condensates

In evaluating DEA, the IARC Working Group also considered three dermal application carcinogenicity bioassays in $B6C3F_1$ mice and Fischer F344/N rats of fatty acid DEA condensates conducted by NTP. These studies involved coconut oil acid DEA condensate (NTP, 1997b), lauric acid DEA condensate (NTP, 1997c), and oleic acid DEA condensate (NTP, 1997d). After careful deliberation, the IARC Working Group concluded that the fatty acid condensate bioassays could not be used for the evaluation of DEA carcinogenicity and did not represent confirmation of the DEA findings.

There are several reasons why the results from these studies cannot be applied to the assessment of DEA. Each of the 3 condensates was a mixture of uncertain and complex composition. Because the test condensates were mixtures, any positive results could not necessarily be ascribed to DEA alone. The actual content of DEA in the mixtures had not been measured by NTP in any of the 3 studies, and the concentrations of free DEA cited in the NTP reports appeared to be estimates based on information provided by the manufacturers. The Working Group also noted differences

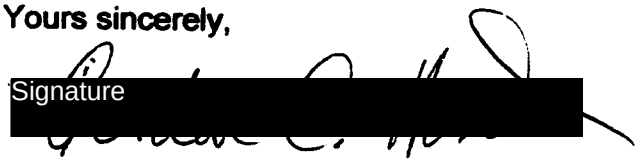
in the DEA concentrations stated in the draft NTP reports compared to the concentrations noted in the final NTP reports on the 3 condensates. In the cases of lauric acid and oleic acid DEA condensates, this change represented a major reduction from approximately 5% to 0.83% and 7.3% to 0.19%, respectively. In addition, when nitrosamine quantitation was carried out by high-performance liquid chromatography (HPLC), the known genotoxic carcinogen, N-nitrosodiethanolamine, was detected at concentrations of 219 ppb in the coconut oil acid DEA condensate, 3,600 ppb in the lauric acid DEA condensate, and at 68 ppb in the oleic acid DEA condensate. Finally, these studies were not designed with the intention of serving as conventional carcinogenicity bioassays of DEA; therefore they cannot be used for this purpose.

Male and Female results in Carcinogenicity Bioassays

Experimental convention and statistical logic determine that the male and female findings within a single species in carcinogenicity bioassays are treated as part of the same bioassay and not as two separate studies. This is the standard approach promulgated by authoritative guideline-setting bodies including IARC and the U.S. Environmental Protection Agency (EPA). It is also consistent with Proposition 65 guidelines. Consequently the gender components of the NTP mouse carcinogenicity bioassay of DEA (NTP, 1997a) should not be considered as independent studies.

I trust this information will help to provide a balanced perspective in the assessment of DEA.

Yours sincerely,



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Tab 2

DIETHANOLAMINE: A CONVERSATION WITH OEHHA STAFF*

by W. T. Stott, Ph.D.

Several topics central to this discussion are listed below with supporting information.

1) The liver tumor response reported in the National Toxicology Program sponsored bioassay of diethanolamine (DEA) reflected a normal spectrum of tumor types and incidence (Text Table A).
   a) Liver tumor development in rodents is a continuum. Hepatocellular adenomas may progress into the less differentiated hepatocellular carcinomas which may progress into the even less differentiated hepatoblastomas.
      i) Dr. Gordon Hard (Pathologist with the American Health Foundation and Chairman of the IARC Carcinogenesis Section, Monograph Working Panel) has stated[1], "it has long been accepted by pathologists with an intimate working knowledge of rodent liver carcinogenesis that liver cancer develops according to a morphologic sequence of lesions. In the continuum, adenomas and carcinomas are sequential stages, with adenomas preceding and being able to progress into carcinomas". "Hepatoblastomas can be regarded as one extreme of this continuum".
      ii) Similar conclusions have been published by a long list of noted pathologists including Dr. J. Popp, formerly head of Pathology at CIIT, and Dr. J. Ward of NCI[2]
      iii) Dr. J. R. Hailey of NTP stated during the review of the DEA Bioassay by the Technical Reports Review Subcommittee that the hepatoblastomas observed[3] "appears to be part of the spectrum of the progression of liver neoplasms in the mouse; as such, with the higher background rate of liver neoplasms in mice, there is a concomitant increase in the incidence of hepatoblastoma."

---

[1] G. Hard (2000). Letter to the Alkanolamines Panel of the American Chemistry Council, September 14, 2000.

[2] International Expert Advisory Committee to The Nutrition Foundation (1983). The Relevance of Mouse Liver Hepatoma To Human Carcinogenic Risk. A Report of The IEAC, The Nutrition Foundation, Inc., ISBN 0-935368-37-X.
Koen, H., Pugh, T. D., Goldfarb, S. (1983). Hepatocarcinogenesis in the mouse. Combined morphologic-stereologic studies. *Am. J. Pathol. 112*, 89-100.
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* This document is identical to the one handed out at the Alkanolamines Panel's September 18, 2000, meeting with OEHHA, except for minor technical corrections.

Text Table A. Liver Tumor Response in the NTP Diethanolamine Bioassay[3]

		Ethanol Controls	40 mg/kg/day	80 mg/kg/day	160 mg/kg/day
Females	Adenomas	32/50	50/50	48/50	48/50
	Carcinomas	5/50	19/50	38/50	42/50
	AD + CA	33/50	50/50	50/50	50/50
Males	Adenomas	31/50	42/50	49/50	45/50
	Carcinomas	12/50	17/50	33/50	34/50
	Hepato-blastmas	0/50	2/50	8/50	5/50
	Total Tumor-Bearing Animals	39/50	47/50	50/50	49/50

b) The incidence of tumors in ethanol-treated controls was unusually high: 64% adenomas and 10% carcinomas in females and 62% adenomas and 24% carcinomas in males thus setting the "baseline" incidence relatively high in the absence of DEA treatment.
   i) Response in females was outside the NTP historical control incidences.
   ii) A tumor promoter would be expected to increase both the number and severity of the "baseline" tumor incidence and type. Dr. G. Hard has further commented on the significance of the high spontaneous tumor incidence of the B6C3F1 mouse model used by NTP[1]: "High spontaneous incidence means that many liver cells are initiated inherently on the pathway to cancer, and a chemical compound can then enhance the clonal expansion of these initiated cells, enhancing in turn, further along the continuum, the sequential conversion of adenomas to a higher grade of neoplastic lesion, i.e., to carcinoma – a mechanism which explains the compound's apparent carcinogenicity."

c) The ethanol-DEA test material acted to promote tumor development in the DEA bioassay.
   i) Due to its genetic predisposition to develop liver tumors, the B6C3F1 mouse carcinogenesis model cannot distinguish between promoters and carcinogens.
   ii) DEA appears to promote liver tumor development in the B6C3F1 mouse model via chronic increases in hepatocellular S-phase DNA synthesis (i.e., cell proliferation; Text Figure A) which has been observed in liver of B6C3F1 mice administered tumorigenic dosages of DEA[4].
   iii) IARC[5] considered the increase in tumor incidence to be only "limited evidence of carcinogenicity"[1] based upon:

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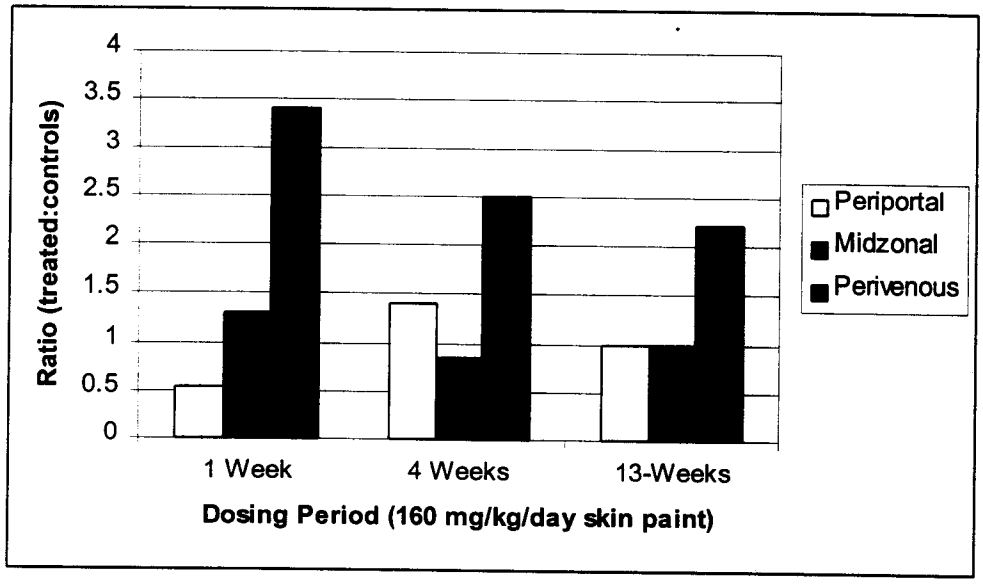
[4] Gembardt, C.et al. (2001). DIETHANOLAMINE (DEA) – SUSTAINED INCREASE IN CELL PROLIFERATION IS RESTRICTED TO TARGET CELLS IN LIVER AND KIDNEYS. Submitted abstract for Ann. Mt. Soc. Toxicol., San Francisco, CA.
[5] International Agency for Research on Cancer, Lyon, France.

(1) High spontaneous incidence of liver tumors in B6C3F1 mice and thus high susceptibility to chemical-induced tumor formation.
(2) Unusually high spontaneous tumor incidence in the controls of the NTP DEA bioassay.

Text Figure A. S-Phase DNA Synthesis in Liver of B6C3F1 Mice Administered a Tumorigenic Dosage of DEA via Skin Painting[4].



2) Neither the result in the male and female mice as part of the NTP DEA bioassay nor those from the DEA-condensate bioassays represent confirmation of the DEA bioassay conclusions.
   a) To avoid bias, experimental confounding factors and false positive results, findings in male and female test animals from a traditionally designed bioassay are considered two parts of a single bioassay and not "independent" studies.
      i) Organizations charged with having to judge bioassay findings have laid out criteria. As an example, guidelines prepared by IARC for use by their Monograph Working Group[6] state that categorization of a chemical as having "Sufficient evidence of carcinogenicity" to require a tumorigenic finding in "(a) two or more species of animals or (b) in two or more independent studies in one species carried out at different times or in different laboratories or under different protocols".
      ii) Guidelines governing listing of "Chemicals Formally Identified by Authoritative Bodies"[7] state a tumorigenic response must be found "in multiple species or strains, in multiple experiments (e.g., with different routes of administration or using different dose levels), or, to an unusual degree, in a single experiment with regard to high incidence, site or type of tumor, or age at onset".

[6] International Agency for Research and Cancer (1999). Evaluation. Monograph Evaluation, Chapter 12, [http://193.51.164.11//Monoeval/Eval.html].
[7] Title 22, Subdivision 1., Chapter 3., Article 3., 12306 (e) (2).

iii) Dr. G. Hard who has chaired several Carcinogenesis Subcommittees of the IARC Monograph Working Groups has stated that "Experimental convention and statistical logic determine that the male and female findings within a single species in carcinogenicity bioassays are treated as part of the same bioassay and not as two separate studies"[1].

b) The bioassays of the fatty acid DEA (amide) condensates; oleiamide, cocamide and lauramide were bioassays of complex mixtures and not of a relatively pure chemical (Text Table B)[8]. A definitive answer regarding the contribution of DEA to tumor formation in these bioassays has not been possible for the following reasons:

i) Unknown compositions especially in the case of oleamide and cocamide test materials in which "unknown organic impurities" and a variety of amides make up a majority of the test materials.

ii) Uncertainties over the concentration of free DEA in the amide-condensates (i.e., the actual dosage of free DEA in these studies was not known).

iii) The lack of a consistent spectrum of toxicity *in vitro* and *in vivo* for the different amide-condensates and DEA suggest a more complex mode of action of condensates.[8,9]

iv) The IARC Working Group evaluating the carcinogenicity data for DEA "concluded that the fatty acid condensate bioassays could not be used for the evaluation of DEA carcinogenicity and did not represent confirmation of the DEA findings".[1] This conclusion was based upon:

(1) Condensates were mixtures of uncertain composition and therefore "any positive results could not necessarily be ascribed to DEA alone".[1]
(2) Actual DEA content was not directly measured.
(3) Presence of nitrosamines in the condensate test materials.
(4) Condensate bioassays were not designed to bioassay the carcinogenic potential of DEA and "therefore they cannot be used for this purpose"[1].

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[8] National Toxicology Program (1997). Toxicology and Carcinogenesis Studies of Coconut Oil Acid Diethanolamine Condensate (CAS No. 68603-42-9) in F344/N Rats and B6C3F1 Mice (Dermal Studies). NTP TR 479, NIH Publication No. 97-3969 (Draft).
National Toxicology Program (1999). Toxicology and Carcinogenesis Studies of Lauric Acid Diethanolamine Condensate (CAS No. 120-42-1) in F344/N Rats and B6C3F1 Mice (Dermal Studies). NTP TR 480. National Toxicology Program (1999). Toxicology and Carcinogenesis Studies of Oleic Acid Diethanolamine Condensate (CAS No. 93-83-4) in F344/N Rats and B6C3F1 Mice (Dermal Studies). NTP TR 481.

[9] Spalding, J. W. et al. (2000). Responses of transgenic mouse lines p53+/- and Tg.AC to agents tested in conventional carcinogenicity bioassays. *Toxicol. Sci. 53*, 213-223.
National Toxicology Program (1999). Toxicology and Carcinogenesis Studies of Diethanolamine (CAS No. 68603-42-9) in F344/N Rats and B6C3F1 Mice (Dermal Studies).. NTP TR 478, NIH Publication No.99-3968.

Text Table B. Condensate Amides Composition and Selected Toxicity Data[8,9].

STUDY	% DEA Reported	Test Material Purity (%)	% "Unknown Organic Impurities"	NDELA (ppb)	Significant Toxicity Data
Oleamide Study [TR 481]	0.19 ["based on the amine value supplied by the manufacturer"]	48	22 [another 30% as unknown fatty acid amides]	68	B6C3F1 (M&F): No increased incidence of liver tumors at ≤30 mkd. Tg.AC Transgenic Mouse Model: Negative at approx. 40 mkd.
Lauramide Study [TR 480]	0.83 & 5 [both values appear based upon manufacturers information]	90	9.2	3600	B6C3F1 (F): Numerical increased incidence of liver tumors at ≥100 mkd. No dose-response. CHO SCE Assay: Positive. Mouse MN Test: Negative at 800 mkd. Tg.AC Transgenic Mouse Model: Positive at approx. 400 mkd.
Cocamide Study [TR 479]	4-8.5 & 19.6 [latter value "based on calculations using the amine value supplied by the manufacturer"]	Identified 40% as Lauramide, Remainder mixed amides.	Not Reported	218	B6C3F1 (M&F): Increased incidence of liver tumors at ≥100 mkd. B6C3F1 (M): Increased incidence of kidney tumors at 200 mkd. CHO SCE Assay: Negative. Mouse MN Test: Positive at 800 mkd. Tg.AC Transgenic Mouse Model: Negative at approx. 250 mkd.
DEA Bioassay [TR 478]	>99% [analysis]			<LOD of 1000 ppb	B6C3F1 (M&F): Increased incidence of liver tumors at ≥40 mkd. B6C3F1 (M): Increased incidence of kidney tumors at ≥40 mkd. CHO SCE Assay: Negative. Mouse MN Test: Negative at 1250 mkd. Tg.AC Transgenic Mouse Model: Negative at approx. 800 mkd.

3) The ethanol vehicle used in the NTP bioassay is a confounding factor in the interpretation of results.
   a) The bioassay involved the dosing of two chemicals, not just one, for the following reasons. Skin painting application of the test material in the absence of restricted access to the application site allowed grooming and subsequent ingestion of both DEA and the ethanol vehicle.
      i) Grooming activity has been reported immediately following application of an ethanol-DEA solution to the skin of a mouse.[10]
      ii) Blood levels of DEA in mice administered DEA dermally with access to the application site are approximately 35% higher than in mice prevented access to the site, proof that some ingestion was occurring.
      iii) The small amount of water in a 95% ethanol solution retards evaporation of ethanol from the skin of a mouse.
   b) A relatively high dosage of ethanol was delivered to mice (Text Table C).

Text Table C. Calculated dosages of ethanol vehicle delivered mice of varying body weights (Equation: Dose (mg/kg/day) = (volume delivered x density (0.816) / Body Wt.).

	Body Weight (Kg)			
	0.03	0.04	0.05	0.06
Males				
Volume/Mouse (uL)				
Min. 41	1115.2	836.4	669.1	557.6
Max. 93	2529.6	1897.2	1517.8	1264.8
Females				
Volume/Mouse (uL)				
Min. 34	924.8	693.6	554.9	462.4
Max. 91	2475.2	1856.4	1485.1	1237.6

   c) Ethanol causes the loss of choline, thus exacerbating the mechanism by which DEA is believed to cause tumors, choline deficiency.
      i) Ethanol increases the rate of choline uptake (demand) by the liver of rats (Text Table D).[11] Increased uptake equates with increased metabolic utilization.
      ii) Ethanol appears to deplete choline via increased betaine oxidase metabolism of choline resulting from increased demand for methionine formation (Text Figure B).[12]

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[10] Stott, W. T. et al. (2000). Potential mechanisms of tumorigenic action of diethanolamine in mice. Toxicol. Lett. 114, 67-75.

[11] Barak, A. J. et al. (1973). Relationship of ethanol to choline metabolism in the liver: A review. Am. J. Clin. Nutr. 26, 1234-1241.

Tuma et al. (1973). Possible interrelationship of ethanol metabolism and choline oxidation in the liver. Can. J. Biochem. 51, 117-120.

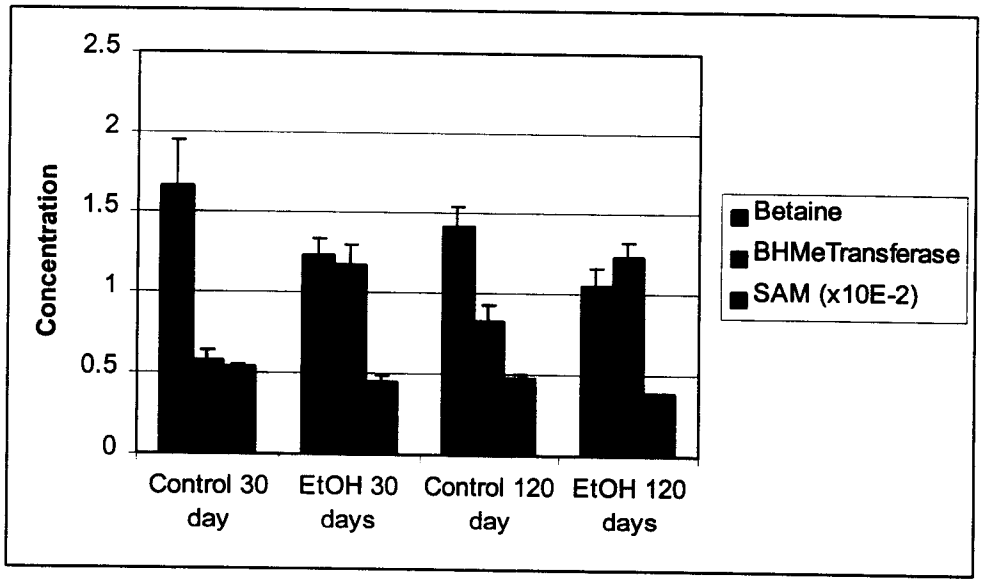
Thompson, J. A. and Reitz, R. C. (1976). Studies of the acute and chronic effects of ethanol ingestion on choline oxidation. Ann. JY Acad. Sci. 273, 194-204.

iii) Guinea pigs were much less susceptible to ethanol-induced depression of liver choline than rats which correlates with their lower betaine oxidase activity (see 4.e.iii(2)).[12] Animals having high choline oxidase activity (e.g., rodents) are likely "much more susceptible to possible ethanol-induced lipotropic [e.g., choline] deficiency than others".[12]

Text Table D. Effect of Ethanol on Choline Uptake in Isolated Perfused Rat Liver[13].

	Choline Uptake
Control	10.5 ±0.71 mg/gram protein
Ethanol Fed	14.9 ±0.74 mg/gram protein
Control	8.5 ±0.71 mg/gram protein
Choline Deficient Diet	10.6 ±0.84 mg/gram protein
Control	Est. 1.7 ±0.0.3 mg/gram liver
Choline Oxidase Inhibitor Added to Perfusate	Est. 0.7 ±0.02 mg/gram liver

Text Figure B. Impact of Ethanol Feeding on Methylation Maintenance in Rats.[14]



[12] Barak, A. J. et al. (1985). Ethanol, the choline requirement, methylation and liver injury. *Life Sci. 37*, 789-791.
Tuma, D. J. et al. (1973). Possible interrelationship of ethanol metabolism and choline oxidase in liver. *Can. J. Biochem. 51*, 117-120.

[13] Barak, A. J. et al. (1971). Ethanol feeding and choline deficiency as influences on hepatic choline uptake. *J. Nutr. 101*, 533-538.

[14] Barak, A. J. et al. (1986). Effects of prolonged ethanol feeding on methionine metabolism in rat liver. Biochem. Cell Biol. 65, 230-233.

4) A well-defined mode of tumorigenic action based upon choline deficiency has been identified for DEA. [15]
   a) DEA has been shown to cause decreases in intracellular choline pools in cultured mammalian cells and in rodents.
      i) *In vitro*, DEA has been shown to competitively inhibit choline uptake in treated Chinese Hamster Ovary cells and Syrian Hamster Embryo primary cultures.
      ii) *In vivo*, DEA has been shown to cause as much as an 85% depression in the primary choline pool, phosphocholine, in mice administered a reported carcinogenic dosage of DEA for 2 or 4 weeks (Text Figure C).
   b) Chronic choline deficiency has been linked to liver tumor formation in rats and mice in a number of studies.[16] The mechanism appears to involve:
      i) A shift in second messenger stimulated chronic activation of protein kinase C isoforms (PKC) with subsequent chronic elevation in hepatocellular and renal cell S-phase DNA synthesis.
      ii) Levels of the important endogenous methylating agent S-adenosylmethionine (SAM) were observed to be depressed in mice administered a reported carcinogenic dosage of DEA (Text Figure D).[15] Hypomethylation of DNA has also been associated with longer-term choline deficiency.
      iii) Alterations in sensitivity to apoptosis regulation.
      iv) Enhanced S-phase DNA synthesis in liver and kidney (see Text Figure A) which is central to most, if not all, nongenotoxic mechanisms of action.[4,17]

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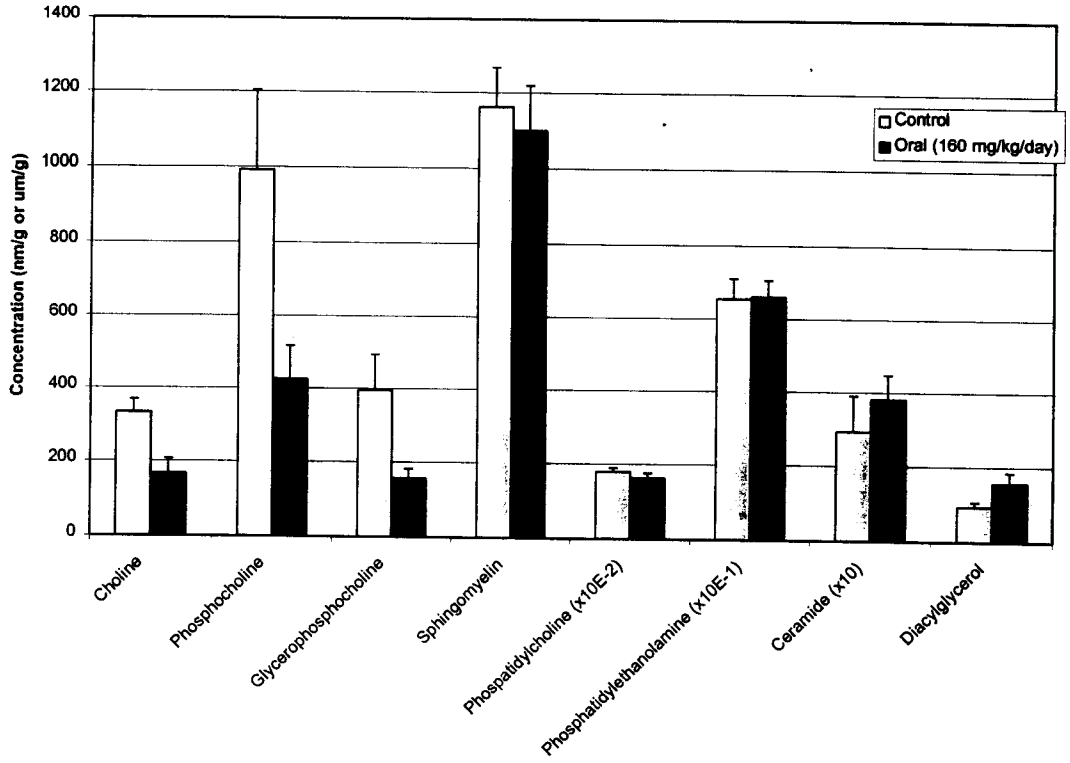
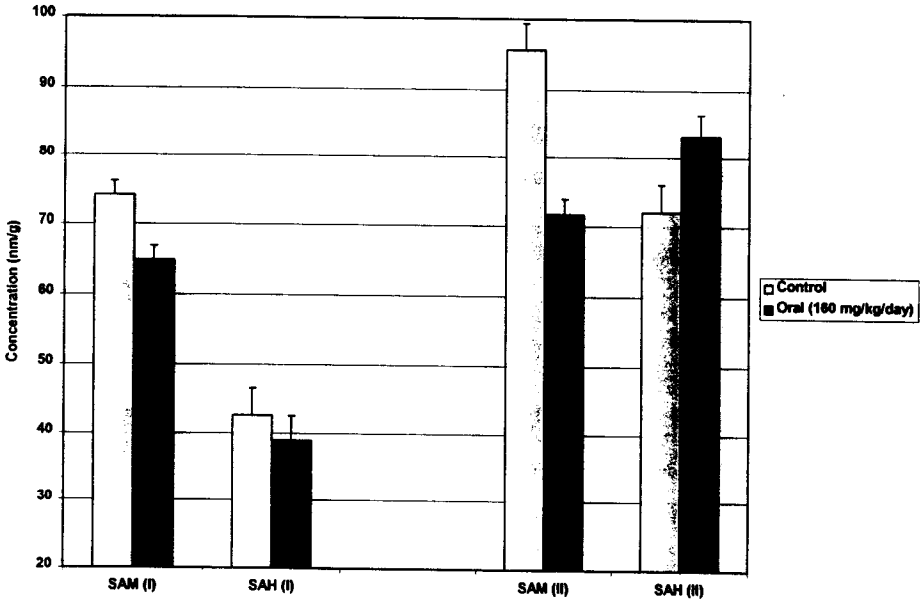
[15] Lehman-McKeeman, L. D. and Gamsky, E.A. (2000). Choline supplementation inhibits diethanolamine-induced morphological transformation in Syrian Hamster embryo cells: Evidence for a carcinogenic mechanism. *Tox. Sci.* 55:303-310.
Lehman-McKeeman, L. D., unpublished data.
Stott, W. T. et al. (2000). Potential mechanisms of tumorigenic action of diethanolamine in mice. Toxicol. Lett. 114, 67-74.
Stott, W. T. et al. (2000). Potential mechanisms of tumorigenic action of diethanolamine in mice. The Toxicol. 54, Abstr. No. 1022.
[16] Zeisel, S. H. and Blusztajn, J. K. (1994). Choline and human nutrition. Ann. Rev. Nutr. 14, 269-296 and references contained within.
Zeisel, S. H. et al. (1995). Choline and hepatocarcinogenesis in the rat. Adv. Exptl. Med. Biol. 375, 65-74 and references contained within.
[17] Butterworth, B. E., Slaga, T. J., Farland, W. and McClain, M. (1991). Chemically-Induced Cell Proliferation. Implications for Risk Assessment. Wiley-Liss, New York.
Goodman, J. I. et al. (1991). Mouse liver carcinogenesis: Mechanisms and relevance. *Fundam. Appl. Toxicol. 17*, 651-665.

Text Figure C. Liver choline, choline metabolites and phospholipids from mice administered a tumorigenic dosage of DEA for 4 weeks.[15]



Text Figure D. S-Adenosylmethione and S-Homocysteine content of mice administered a tumorigenic dosage of DEA for 4 weeks. [15]



c) Direct evidence of the involvement of choline deficiency in DEA-induced tumorigenic activity has come from an *in vitro* cell transformation assay that is purported to detect nongenotoxic carcinogens. Supplementation of the media with choline by Lehman-McKeeman et al.[15] inhibited DEA-induced morphological transformation of the cells (i.e., resulted in a negative test response).

d) It has been suggested that DEA tumorigenesis may be related to the metabolic incorporation of DEA into phospholipids. Data accumulated to-date, however, do not support this:

   i) There has been evidence of incorporation of DEA altered phospholipids in rats at the dosages tested in the NTP bioassay yet no tumors were noted in these animals.[18]
      (1) Male and female F344/N rats administered 63 and 32 mg/kg/day DEA, respectively, via their drinking water for 13 weeks have microcytic anemia and metabolites of DEA accumulate in RBCs of rats.
      (2) Erythrocytes undergo changes in cell shape upon alterations in membrane phospholipids resulting in loss of RBCs and development of anemia.
   ii) The regional location of increased S-phase DNA synthesis, cell turnover, in the liver of mice administered DEA over 3-7 day period is not consistent with the normal location of hepatocyte regeneration in the lobule. Newly synthesized hepatocytes would have the highest level of DEA-altered membrane phospholipids.[4]
   iii) Disruption of gap junctional intercellular communication (GJIC) may lead to uncontrolled growth of cells and ultimately tumors.[19] Disruptions in membrane function due to incorporation of aberrant lipids would be expected to alter GJIC. However, preliminary experiments have indicated no interruption in GJIC in mice administered a tumorigenic dose level of DEA for 30 days.[20]

e) The results of numerous studies indicates that humans are resistant to the development of choline deficiency relative to rodents. Subsequent studies, planned or in progress, will directly address interspecies differences in sensitivity (see below under "Planned Research"). Significant findings to-date include:
   i) Evidence of choline deficiency has been found in humans under extreme conditions that preclude chronic situations necessary to pose a tumorigenic risk.
      (1) Choline deficiency has been observed in patients suffering malnutrition and liver cirrhosis and thus compromised ability to synthesize choline, or

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[18] Melnick, R. L et al. (1994). Toxicity of diethanolamine. 1. Drinking water and topical application exposures in F344 rats. *J. Appl. Toxicol. 14*, 1-9.
J. Waechter et al., Unpublished data.
Kuypers, F. A. et al. (1984). The membrane of intact human erythrocytes tolerates only limited changes in the fatty acid composition of its phosphatidylcholine. *Biochim. Biophys. Acta 769*, 337-347.
Kuypers, E. W. et al. (1985). Survival of rabbit and horse erythrocytes in vivo after changing the fatty acyl composition of their phosphatidylcholine. *Biochim. Biophys. Acta 819*, 170-178.
[19] Klaunig, J. E. and Ruch, R. J. (1990). Biology of disease. Role of inhibition of intercellular communication in carcinogenesis. *Lab. Investig. 62*, 135-146.
[20] Stott, W. T., Unpublished data.

undergoing long-term total parenteral feeding.[21] These changes were readily reversible upon providing choline or lecithin supplementation.

(2) Prolonged fasting of healthy subjects resulted in only modest changes in plasma choline levels and no evidence of hepatic injury.[22]

ii) Nonhuman primates reportedly were much more resistant to development of choline deficiency related liver pathology than rats.

iii) An enzymological basis for at least some of the resistance of higher species to development of choline deficiency has been identified.

(1) As noted, choline may undergo oxidation via a well established pathway to produce betaine which is instrumental in methionine synthesis.

(2) Choline oxidation occurs at a much higher rate in rodents than higher mammals, including humans.[23]

5) An ongoing research program is being sponsored by ACC which is designed to provide:

a) Data upon sex differences in mice, dose-response and the impact of choline supplementation in liver and kidney of DEA treated mice utilizing S-phase DNA synthesis rates as the principle measured parameter. The proposed work will include:

i) Evaluation of sex-differences and the reversibility of effects of DEA in liver and kidney of mice.

ii) Dose-response of effects of DEA in liver and kidney of male mice.

iii) Evaluate the effects of choline supplementation upon liver and kidney of male mice. Elimination of synthesis activity by choline supplementation will provide a direct link between choline deficiency and a significant component of nongenotoxic carcinogenesis.

b) Data upon species differences, including humans, in response to DEA treatment using cultured primary hepatocytes. Hepatocytes have demonstrated their usefulness in examining interspecies differences in sensitivity to chemicals using a number of endpoints associated with nongentoxic carcinogenesis. The proposed work will include:

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[21] Chawla, R. K. et al. (1989). Choline may be an essential nutrient in malnourished patients with cirrhosis. *Gastroenterology 97*, 1514-1520.
Buchman, A. L. et al. (1992). Lecithin increases plasma free choline and decreases hepatic steatosis in long-term total parenteral nutrition patients. *Gastroenterology 102*, 1363-1370.
Buchman, A. L. et al. (1993). Low plasma free choline is prevalent in patients receiving long term parenteral nutrition and is associated with hepatic aminotransferase abnormalities. *Clin. Nutr. 12*, 33-37.

[22] Savendahl, L. et al. (1997). Prolonged fasting in humans results in diminished plasma choline concentrations but does not cause liver dysfunction. *Am. J. Clin. Nutr. 66*, 622-625.

[23] Hoffbauer, F. W. and Zaki, F. G. (1965). Choline deficiency in baboon and rat compared. *Arch. Pathol. 79*, 364-369.
Wilgram, G. F. et al. (1958). Kwashiorker type of fatty liver in primates. *J. Exp. Med. 103*, 361.
Sideransky, H. and Farber, E. (1960). Liver choline oxidase activity in man and in several species of animals. *Arch. Biochem. Biophys. 87*, 129-133.
Haubrich, D. R. and Gerber, N. H. (1981). Choline dehydrogenase. Assay, properties and inhibitors. *Biochem. Pharmacol. 30*, 2993-3000.

i) Examination of DEA-induced changes in S-phase DNA synthesis rates.
ii) Evaluation of gene expression patterns. Modification of gene expression is central to the action of hepatic tumor promoters and specific patterns of genes being expressed may provide a "signature" of DEA-induced changes that may be evaluated across species.
iii) Evaluation of the methylation status of DNA.
iv) Effects of treatment on GJIC.

Tab 3

Ernest E. McConnell, D.V.M., M.S. (Path), DACVP, DABT
President, ToxPath, Inc.

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919-848-1576

3028 Ethan Lane
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Raleigh, NC 27613

28 May 2001

To: James G. Keay, Ph.D
Vice President Business Director, Bulk Pyridines
300 North Meridian Street, Suite 1500
Indianapolis, IN 46204

Subj: NTP Pyridine Study

Per your request, I have reviewed the subject study and offer the following comments:

I will concentrate my comments on the mouse portion of the study because that is the one that is most flawed and of most import for consideration by CAL EPA. The major flaw in the mouse portion of the study was that even the lowest exposure/dose clearly exceeded the Maximum Tolerated Dose (MTD), using contemporary definitions of the term, and was conducted in an abnormal animal model ("fat mouse"). I base this view on the following observations:

First, the only lesion of concern in pyridine exposed mice is the presence of an increased incidence of specific types of liver tumors, e.g. hepatocarcinomas and hepatoblastomas. But interestingly, the total tumor response in the liver shows **no dose response**; i.e. the tumor response was essentially flat across the exposure groups of both sexes.

Second, from an apparently well-conducted toxicokinetic study conducted by Mason laboratories, the metabolism/excretion of pyridine appears to be saturated at the doses selected for the 2-year bioassay [TSI Mason Laboratories, Single Dose Toxicokinetic Study of Pyridine (CAS #110-86-1) in B6C3F1 Mice, Report No. MLI-NTP-20-91-119, TSI Mason Laboratories (May 20, 1992)]. This study was started after the NTP 2-year bioassay was initiated and appears to not have been available to help select doses for the cancer bioassay.

Third, it is apparent that the control mice in this study were clearly "*abnormal*" in terms of other control groups of B6C3F1 mice used by the National Toxicology Program in the past. The tumor incidence in male mice in this study was 76% and in females 84%. That compares with a historical incidence in NTP oral studies of 42% (range = 10-68%) in males and 24% (range = 6-56%) in females (Haseman et al., 1999, Pathology of the Mouse, pp. 679-689). However, in six contemporary water studies to this one a much higher incidence of liver tumors was observed, e.g. 74% (range = 53-81%) for males and 63% (range = 32-84%) for females. Clearly, the spontaneous incidence of liver tumors has increased over time. But even more impressive is the

fact that the liver tumor incidence in control B6C3F1 mice where water was used as the vehicle is even higher during the same time frame than that for other routes of exposure used by the NTP. For example in oral feed studies the rate for males was 52 ± 8% and females = 32 ± 10%; corn oil gavage in males was 52 ± 15% and females = 27 ± 14%; and in inhalation studies in males was 52 ± 17% and females = 31 ± 11%.

Fortunately, the explanation for the increasing incidence over time and in this particular study is quite clear. It appears to be directly related to the weight of the mice during the study. For example, during my years (1980s) at the NTP the average maximum weight for both male and female mice was in the range of 30-35 g. The mean weekly body weight of mice in the pyridine study was 55 g for males and 63 g for females or about double the weight during the 1980s. Also, the body weight in this water study was much higher that in dosed-feed or inhalation studies during the same time period as shown in the below Table. This Table is derived from the following reference: TDMS, 1998, Tumor Incidence in Control Animals by Route and Vehicle Administration: B6C3F1 Mice, OCR Services, PO Box 12510, RTP, NC 27709. Some of this causal argument is described in the Haseman chapter referenced above.

Body Weight Versus Liver Tumor Incidence

	Male Mice		Female Mice	
	Tumor (%)	Body Weight	Tumor (%)	Body Weight
Pyridine	76	55	84	63
Oral water studies n = 6	74 ± 11	52	63 ± 22	58
Oral feed studies n = 17	52 ± 8	55	32 ± 10	52
Inhalation studies n=19	52 ± 17	48	31 ± 11	49

However, the relation between body weight (BW) and the incidence in liver tumors becomes even more apparent when one compares the three studies with the lowest BW to the liver tumor incidence in three highest BW studies as shown in the below Table.

Body Weight (bw) Versus Liver Tumor Incidence in Mice from the Three Highest and Three Lowest BW Studies

	Male Mice		Female Mice	
	Tumor (%)	Body Weight	Tumor (%)	Body Weight
Pyridine	76	55	84	63
Oral water studies (low bw)	67	50	47	55
(high bw)	79	54	78	61
Oral feed studies (low bw)	44	46	22	46
(high bw)	59	52	43	58

Inhalation studies (low bw)	27	41	27	39
(high bw)	58	52	39	58

While I didn't do any statistics on the above table, I think that the correlation of body weight and liver tumor incidence is indisputable! In fact, the high background incidence of liver tumors has developed into such a problem that the NTP has "rederived" its' stock of B6C3F1 mice using frozen embryos stored from the mid-1980s when the incidence of liver tumors was much lower.

This finding is further and dramatically substantiated by the results of the diet restriction studies conducted by the NTP (Toxicology and Carcinogenesis Studies of Pyridine (CAS No. 110-86-1) in F344/N Rats and B6C3F1 Mice (Drinking Water Studies), TR 460, 1997). The impetus for this study was the findings from the National Center for Toxicology Research (NCTR) research into the relationship of body weight and longevity. NCTR had clearly shown that diet restriction (DR) resulting in lower body weights directly affected survival (increased) and other parameters including the spontaneous incidence of several types of tumors. With this background the NTP designed a study to see the effects of diet restriction with two chemicals in a standard feeding bioassay. Included in this study were male mice exposed to the chemical salicylazosulfapyridine (SASP) and male and female mice exposed to scopolamine. Interestingly, there were three control groups in these studies, one fed *ad libitum*, one with a diet restriction to match the amount of food consumed by the high dose of the treatment group (DR- 1) and one that was restricted so that the body weight would be 85% of DR-1 (DR 85%). The results of this study on the incidence of liver tumors are shown in the following Table.

Body Weight Versus Liver Tumor Incidence in Diet Restricted Mice

	Male Mice		Female Mice	
	BW (g) 18mo	Tumor %	BW (g) 18 mo	Tumor %
SASP Controls				
Ad libitum	54.4	48	not done	
DR-1	45.3	28	not done	
DR 85%	45.0	35	not done	
Scopolamine Controls				
Ad libitum	52.4	60	55.3	43
DR-1	45.7	20	43.4	18
DR - 85%	38.9	10	34.5	6

Another finding that bears on this issue is the incidence of liver tumors in the high dose scopolamine group of exposed male mice. Scopolamine caused a marked reduction in the body wt of these animals (32.7 g at 18 mo) and reduced the incidence of liver tumors to **2%**! This in spite of the fact that the survival rate of these mice was 96% at the end of the 2-year study as compared to a 70% survival in the pyridine controls. Another interesting aspect of this study was that a further 50 mice in the 85% diet restriction groups were allowed to live to 3 years using the same diet restriction (the *ad libitum* group was not included because they would have all been

dead prior to 36 months). The results of this portion of the study showed that 56% of the males and 40% of the females were still alive. But more importantly, the incidence of liver tumors was still only 26% in males and 30% in females, far below the incidence at 24 months with the *ad libitum* diet.

My interpretation of the above findings is that the mice in the pyridine study were "primed" to develop tumors because genetic (propensity to be overweight) and dietary (high caloric content) factors. In that sense these are "abnormal" animals and the results of bioassays conducted in such models has to be viewed in this context. I am convinced that any chemical fed at levels that exceeded the metabolic threshold and which is metabolized to any extent in the liver would result in an increase in liver tumors in these abnormal mice. It needs to be stressed that pyridine did not cause an increase in the incidence of total liver tumors, but only in carcinomas and hepatoblastomas. Most of the information on body weight and its relationship to liver tumors in mice has been developed after the pyridine bioassay was conducted.

If you have questions concerning this report please feel free to contact me at the above address/ phone.

Signature [redacted]

Ernest E. McConnell

Tab 4

Toxicology Consulting Services
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6905 Earlsgate Way
Rockville, MD 20852

May 30, 2001

Dr. George Alexeeff
OEHHA
CAL-EPA
1515 Clay Street
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Oakland, CA 94612

Dear Dr. Alexeeff,

These comments are being submitted regarding OEHHA's intention to list pyridine on the Proposition 65 list of substances known to the State to cause cancer based solely on the finding of an increase in mouse liver tumors in B6C3F1 mice in an NTP 2-year bioassay study. This decision would rely on the NTP study report as the authoritative body. The comments are being submitted on behalf of Reilly Industries. They represent my own scientific evaluation of the NTP Technical Report No. 470 entitled "Toxicology and Carcinogenesis Studies of Pyridine (CAS No. 110-86-1) in F344/N Rats and B6C3F$_1$ Mice (Drinking Water Studies)" and related scientific information.

It is my opinion that the increase in liver tumors in B6C3F1 mice observed after treatment with pyridine should not be the basis for an NTP authoritative body listing as a substance known to the State to cause cancer. Rather, the study and all of the information and other studies relevant to an evaluation of whether there is sufficient information to list pyridine for Proposition 65 should be fully evaluated by the Carcinogen Identification Committee. I base my opinion on the following facts:

1. **The NTP 2-year mouse bioassay of pyridine was initiated in 1991, before new information was available on the relationship of body weight to the incidence of liver tumors in B6C3F1 mice. The high body weights of the mice confound the study.**

   The 2-year study on pyridine in B6C3F1 mice was initiated in April 1991. Animals were from a Taconic Farms colony of B6C3F1 mice (Germantown, NY). They were given food *ad libitum* and housed in solid cage pans. During the course of the study, the mice became extremely heavy.

   It was known for some time that body weight correlated with the incidence of tumors. In 1994, Turturro *et al.* published a study that showed even small increases in body weights in B6C3F1 mice were directly related to the incidence of liver tumors. In 1995, the National Center for Toxicology Research organized a symposium on Caloric Restriction and Toxicity

and the proceedings were subsequently published (Hart *et al.*, 1995). During this meeting, evidence that body weights in NTP bioassays strongly influenced the liver tumor response was reviewed and discussed. As stated in the proceedings from the symposium, "What is surprising is that it appears that even relatively minor changes in BW correlates well with fairly significant differences in survival at 24 months on test as well as the incidence of selected pathologies, including liver tumors...BW differences as small as 5 g at 12 months on test in male mice results in a doubling in incidence of liver tumors" (p 184, Hart *et al.*, 1995).

A diet restriction study was conducted by NTP to more fully understand the potential for confounding a study by alterations in body weight. This study, which included diet restriction studies on 4 different substances, was reported by the NTP in 1997 (NTP, 1997a). In NTP's words, "Theoretically, comparisons between otherwise identical studies conducted several years apart could yield disparate results influenced primarily by the body weight of the animals." (p 17, NTP, 1997a).

In the diet restriction study in male B6C3F1 mice, salicylazosulfapyridine resulted in an increase in liver tumors when feeding was ad libitum. No such increase in liver tumors occurred in feed restricted animals fed similar doses of the material even when the study was extended for up to 3 years (NTP, 1997a). This new information casts doubts on the significance of an increase in mouse liver tumors when obese animals are studied.

In a recently published book, Pathology of the Mouse, Haseman *et al.* report on the neoplasm incidence in control B6C3F1 mice (Haseman *et al.*, 1999). This chapter discusses the strong influence of body weight on the rate of liver cancer in these animals. "Liver appears to be the only site in B6C3F1 mice that shows a strong correlation between neoplasm incidence and body weight" (p 680, Haseman *et al.*, 1999). Liver tumors in these mice are unique in that regard. A finding of an increase in liver tumors in obese B6C3F1 mice should receive thorough review. This finding should not be assumed to be meaningful for human health, and not be used for listing under Proposition 65 without this review.

The information regarding the relationship between body weights in B6C3F1 mice and liver tumors is directly relevant to pyridine for the following reasons:

- The body weights of control mice in the pyridine study were among the highest observed in any NTP study. Female control mice reached during this study an average of 63 grams, a huge weight for female mice. The relationship of these body weights and the liver tumor response was not fully recognized at the time of the pyridine study.

- The colony of animals that was used in the pyridine study has subsequently been rederived from frozen embryos in part because of the increase in incidence of liver tumors observed in studies like the pyridine study. This new colony was not available at the time the pyridine study was started in 1991 (NIEHS, 2000).

- The incidence of liver tumors in control animals in the pyridine study was among the highest ever observed in an NTP study and for female mice outside the historical control

range for NTP. The incidence of total liver tumors in control males was 76% and control females 84% (NTP, 2000).

A comparison can be made of the NTP historical control data published in the reference book on B6C3F1 mice, to the incidence of liver tumors in control animals in the pyridine study (Haseman *et al.*, 1999). As can be seen from the data in the following table, the control animals in the pyridine study and thus the lot of animals utilized in the pyridine study and the animal housing conditions in the pyridine study resulted in unusually high rates of liver tumors. In particular, the extraordinary rate of liver tumors in control females in the pyridine study would significantly diminish the use of these data for regulatory purposes.

Control Liver Tumor Incidences in B6C3F1 Mice

Liver Tumor	Historical Control Rate (males)*	Pyridine Control Rate (males)**	Historical Control Rate (females)*	Pyridine Control Rate (females)**
Adenoma	29	58	17	76
Carcinoma	18	30	8	27
Hepatoblastoma	0	4	0.1	2

* Haseman *et al.*, 1999.
** NTP, 2000.

- The body of information regarding body weights and liver tumors in B6C3F1 mice was not considered in the pyridine report, as it was not fully available at that time.

- The relationship of mouse liver tumors and body weights as it impacts the pyridine study was not discussed at the NTP Technical Report Subcommittee peer review or taken into account during the peer review at the public meeting as it was not fully available at that time (NTP, 1997b).

- Two transgenic studies in mice have subsequently been completed by the NTP and are both negative (Spalding *et al.*, 2000). The results were not reported in the NTP report or considered by the NTP Technical Report Subcommittee.

- A recent study in B6C3F1 mice treated *in vivo* with pyridine was negative for unscheduled DNA synthesis in the liver. The full report of this GLP compliant study is attached for review (SRI, 2000) and has been published (MacGregor *et al.*, 2000).

The available information on the relationship of body weight and liver tumors in B6C3F1 mice and its impact on the findings from the NTP bioassay should be fully evaluated by the OEHHA Carcinogen Identification Committee.

2. **Toxicokinetic data from studies completed a year after the 2-year study was initiated indicated that the doses of pyridine given to the B6C3F1 mice in the 2-year study were in a range where metabolism and/or excretion were likely saturated. This new information casts questions on whether the 2-year bioassay was in fact conducted on 3 different doses of pyridine and whether the doses administered exceeded the dose that could be metabolized and excreted by the mice.**

- The 2-year bioassay of pyridine was initiated in April 1991. The doses selected were based on the results of the 13-week study in mice finished in 1990 (NTP, 2000). Doses selected and the average mg/kg consumption of pyridine reported in the NTP study are in the following table.

NTP 2-Year Bioassay on Pyridine in B6C3F1 Mice

Study Group	Pyridine in Drinking Water (ppm)	Dose (mg/kg)
$B6C3F_1$ Females	125	15
$B6C3F_1$ Females	250	35
$B6C3F_1$ Females	500	70
$B6C3F_1$ Males	250	35
$B6C3F_1$ Males	500	65
$B6C3F_1$ Males	1000	110

- In 1992, a year after the NTP 2-year bioassay in B6C3F1 mice was started, TSI Mason Laboratories completed toxicokinetic studies on pyridine (TSI Mason Laboratories, 1992a,b). One of the studies was a single dose gavage study in B6C3F1 mice (TSI Mason Laboratories, 1992a). This study demonstrated that a single gavage dose of 10 mg/kg pyridine in males was eliminated from plasma during the 360-minute observation period but a single gavage dose of 10 mg/kg of pyridine in females was not completely eliminated during the 300-minute observation period. After just a single dose of 50 mg/kg of pyridine, plasma levels of male and female mice remained nearly unchanged over the 400-minute period. Since this was only a single dose study, it is not known how much pyridine plasma levels would rise after daily chronic exposure.

- The conclusion of the TSI Mason Laboratories (1992a) study states on page 14:

  "From the data presented here it is clear that most of the doses used in the chronic study are substantially outside of the linear range. The data do not permit a conclusion as to the upper limit of the linear range."

- A copy of toxicokinetic information on pyridine was obtained from the NTP archives. This includes a full copy of the study in mice (TSI Mason Laboratories, 1992a).

- This new toxicokinetic information was not available to NTP at the time doses were selected for the 2-year bioassay on pyridine.

- The findings from the toxicokinetic studies conducted by TSI Mason Laboratories were not reported in the NTP study report and are new information.

- The findings from the toxicokinetic study were not discussed at the NTP Technical Report Subcommittee review (NTP, 1997b) and are new information.

- Current practice at NTP is to investigate the toxicokinetics of a test material prior to dose selection using the several routes of exposure, including the route selected for the chronic study (Bucher, 2001).

- As stated in the proceedings from a workshop entitled "National Toxicology Program Studies: Principles of Dose Selection and Applications to Mechanistic Based Risk Assessment," toxicokinetic information is used to select doses for chronic bioassays (p 3, Bucher *et al.*, 1996). The top dose is selected to be in the saturating range of a chemical elimination profile, while the two lower doses "are selected that are near, but below, the inflection point of a kinetic curve and well within the range of linear elimination kinetics" (Bucher *et al.*, 1996).

- Two of the 3 doses of pyridine for both males and females clearly exceeded these NTP criteria. From the available information in the toxicokinetic studies, it is not clear if the top dose exceeded the criteria.

- This directly affects the NTP study as it cannot be assumed to be a study of 3 different doses of pyridine if metabolism and/or elimination were saturated.

- Saturation of metabolism and/or elimination creates in effect artificial conditions within the animal. This is important information that directly impacts whether the oncogenicity observed has any relevance.

- The increase in mouse liver tumors observed in the NTP study should be viewed in context of the available toxicokinetic information.

- The available toxicokinetic information and its impact on the findings from the NTP bioassay should be fully evaluated by the OEHHA Carcinogen Identification Committee.

3. **While the NTP report states there is clear evidence of carcinogenicity in the liver of B6C3F1 mice, this is only indicative of an increase of tumors at that site in that study. The NTP report is not making a statement regarding the carcinogenicity classification of the chemical, and is not conducting a full evaluation of the data relevant to the**

carcinogenicity classification when it determines if there is a site-specific increase in tumors that is "clear evidence." Further, there is no indication in the study report that NTP was implying it was doing this kind of evaluation. NTP management should be directly consulted on this point as I believe the conclusions from their studies are being misinterpreted in the authoritative body process for listing under Proposition 65.

4. **There is no indication that the 2-year bioassay of pyridine in B6C3F1 mice is considered by NTP to be two independent studies -- one on each sex. In fact, in my opinion, NTP fully recognizes that many factors are study-specific and would affect both male and female animals similarly. Therefore, each NTP study on a single species cannot be considered to be two independent studies routinely. NTP management should be directly consulted on whether they believe that the chronic bioassay on each sex of one species should be considered two independent studies on a routine basis. I believe their studies are being misinterpreted in the authoritative body process for listing under Proposition 65.**

With respect to the pyridine study, specifically, there are several factors that support the finding that the studies were not independent studies:

Source of the animals: The animals, both males and females, were from the same colony of B6C3F1 mice at Taconic Farms, NY, that have now been replaced.

Dose selection: Doses selected for both male and female mice appear to be too high based on the available toxicokinetic data.

Growth characteristics and body weights: Both male and female growth characteristics in the pyridine study are unusual in that both sexes became extremely heavy for B6C3F1 mice.

The unusually high rate of liver tumors in control animals: The control incidence of mouse liver tumors is extremely high. The incidence of liver tumors in control males was 76% and control females 84% (NTP, 2000). This value is amongst the highest for males and outside the historical rate for females.

The NTP report of the 2-year bioassay on pyridine must not be taken out of context. Based on the new information available after the NTP study was conducted, the NTP report and all of the relevant information regarding the oncogenicity of pyridine should be fully reviewed by the Carcinogen Identification Committee in consideration of whether pyridine should be listed under Proposition 65.

After your review, I would appreciate the opportunity to discuss this information.

Sincerely yours,

Signature [redacted]

Judith A. MacGregor Ph.D., D.A.B.T.
Cc Dr. John Bucher NTP, NIEHS

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Tab 5

of the high-dose male group was lower than that of the vehicle controls from week 8 and the mean body weight of the high-dose female group was lower than that of the vehicle controls from week 97. There were no increases in tumours in treated groups compared with the vehicle controls (National Toxicology Program, 1999a).

3.2 Genetically modified mouse

Groups of 15–20 female Tg.AC mice, which carry a zeta-globin promoted v-Ha-*ras* gene on an FVB background, 14 weeks of age, were administered diethanolamine topically in 95% ethanol (the diethanolamine used was from the same chemical batch as that used in the mouse National Toxicology Program study (National Toxicology Program, 1999a). The diethanolamine was administered in 200-μL volumes, five times per week for 20 weeks. The concurrent negative control groups were treated with 200 μL 95% ethanol. The positive control group was treated with 1.25 μg 12-*O*-tetradecanoylphorbol 13-acetate (TPA; approximately 99% pure) twice per week for 20 weeks. The doses of diethanolamine selected were based on the maximum tolerated dose used earlier (National Toxicology Program, 1999a) and were 5, 10 or 20 mg diethanolamine per mouse per application (higher than the MTD). Survival was high in both the control (90%) and treated groups (80–95%). Lesions were diagnosed as papillomas when they reached at least 1 mm in diameter and persisted for three weeks. Animals that did not survive until the end of week 10 were not included in the data summaries or calculations. Six weeks after the last application, all surviving mice were killed. There was no evidence of chronic irritation or ulceration at the site of application. In contrast to the positive controls, which developed multiple papillomas in 18/20 animals, there was no increase in the incidence of skin tumours in diethanolamine-treated animals in this model (Spalding *et al.*, 2000).

[The Working Group was aware of three carcinogenicity bioassays (dermal application studies) in B6C3F$_1$ mice and Fischer 344/N rats of fatty acid-diethanolamine condensates conducted by the National Toxicology Program. These were coconut oil acid, lauric acid and oleic acid diethanolamine condensates (National Toxicology Program, 1999b,c,d). The same three condensates were also tested in the transgenic Tg.AC and *p53*$^{+/-}$ mouse models (Spalding *et al.*, 2000). The Working Group concluded that these studies could not be used in the evaluation of the carcinogenicity of diethanolamine *per se*. This judgement was based on the fact that the substances tested were complex mixtures of imprecise composition, that the actual diethanolamine content had not been measured in any of the three studies and therefore the precise levels of exposure were indeterminable, and the fact that these studies were not designed as, and did not represent, conventional or adequate carcinogenesis bioassays of diethanolamine.]