



April 6, 2012

Via e-mail to: P65Public.Comments@oehha.ca.gov

Ms. Cynthia Oshita  
California Environmental Protection Agency  
Office of Environmental Health Hazard Assessment (OEHHA)  
1001 I Street  
Sacramento, California 95814

**RE: Notice of Intent to List Diethanolamine under Proposition 65 by the Labor Code Mechanism (OEHHA Notice dated 1/20/2012; California Labor Code 6382)**

Dear Ms. Oshita:

I am writing on behalf of the Alkanolamines Panel of the American Chemistry Council (ACC). The ACC is the major trade association for the chemical producer industry.<sup>1</sup> The ACC Alkanolamines Panel is comprised of companies that produce ethanolamines, including diethanolamine (DEA; CAS # 111-42-2).<sup>2</sup>

This submission is in response to OEHHA's notice of intent to list DEA under Proposition 65 by means of the Labor Code mechanism.<sup>3</sup> OEHHA states that it is evaluating a "ministerial" listing under

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<sup>1</sup> The American Chemistry Council (ACC) represents the leading companies engaged in the business of chemistry. ACC members apply the science of chemistry to make innovative products and services that make people's lives better, healthier and safer. ACC is committed to improved environmental, health and safety performance through Responsible Care<sup>®</sup>, common sense advocacy designed to address major public policy issues, and health and environmental research and product testing. The business of chemistry is a \$720 billion enterprise and a key element of the nation's economy. It is one of the nation's largest exporters, accounting for ten cents out of every dollar in U.S. exports. Chemistry companies are among the largest investors in research and development. Safety and security have always been primary concerns of ACC members, and they have intensified their efforts, working closely with government agencies to improve security and to defend against any threat to the nation's critical infrastructure.

<sup>2</sup> Members of the ACC Alkanolamines Panel include The Dow Chemical Company and BASF (BASF Corporation/BASF SE). Huntsman Corporation also participates with the Panel in research programs. All three companies are producers of diethanolamine (DEA). It should be clarified that US-based BASF Corporation does not produce DEA in the US; but its German-based parent company, BASF SE, does produce DEA in Germany.

<sup>3</sup> On January 20, 2012, OEHHA posted its intentions to list DEA under the Labor Code mechanism on its public website: [http://www.oehha.ca.gov/prop65/CRNR\\_notices/admin\\_listing/intent\\_to\\_list/noil012012.html](http://www.oehha.ca.gov/prop65/CRNR_notices/admin_listing/intent_to_list/noil012012.html); [http://www.oehha.org/prop65/CRNR\\_notices/admin\\_listing/requests\\_info/Ext\\_4LC\\_022112.html](http://www.oehha.org/prop65/CRNR_notices/admin_listing/requests_info/Ext_4LC_022112.html)



the California Labor Code because of the classification of DEA by the International Agency for Research on Cancer (IARC) as a possible human carcinogen.

The ACC Alkanolamines Panel does **not** support such a listing by OEHHA given the current scientific data on DEA. The Panel strongly urges that OEHHA withdraw its intended listing of DEA under the Labor Code mechanism.

### **OEHHA Needs to Consider Various Factors Before Making a Final Listing Decision**

Because of the ministerial listing mechanism, OEHHA states that it will not consider any scientific arguments concerning the weight or quality of the evidence considered by IARC. The ACC Alkanolamines Panel, however, urges OEHHA to consider every IARC monograph and conclusion, as well as other toxicological and mechanistic data, in applying scientific judgment to each specific chemical case, including DEA.

Therefore, OEHHA should wait until the IARC volume 101 monograph addressing DEA is published before making a listing decision, so that it can properly evaluate the full IARC assessment. As of the date of this letter (April 6, 2012), IARC has not published its volume 101 monograph.

OEHHA is not considering a number of factors in using its Labor Code listing of DEA. It should consider these factors, which include:

- previous IARC and OEHHA reviews of DEA;
- a 2006 change in IARC's criteria for classifying chemicals as carcinogens, which are not founded on sound scientific principles of cancer study design, or on Good Laboratory Practices;
- mechanistic studies indicating that the mode of DEA tumorigenesis in mice is not relevant to humans;
- significant limitations of the single NTP chronic mouse study on which OEHHA is indirectly relying upon for its Labor Code listing; and,
- a decision of the National Toxicology Program (NTP) not to include DEA as an agent that is "known or reasonably anticipated to cause cancer in humans" in its Report on Carcinogens, based on its own review of the NTP chronic mouse study of DEA.

These factors are discussed below.

#### A. Previous IARC and OEHHA Reviews of DEA

In 2000, IARC reviewed DEA and concluded that DEA is "not classifiable as to its carcinogenicity to humans" (IARC 2000). Under the IARC categorization scheme at that time, DEA was classified as a "Group 3" substance. The basis for this classification was as follows:<sup>4</sup>

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<sup>4</sup> See IARC Preamble at page 27 (IARC 2000).



This category is used most commonly for agents for which the evidence of carcinogenicity is inadequate in humans and inadequate or limited in experimental animals.

Exceptionally, agents for which the evidence of carcinogenicity is inadequate in humans but sufficient in experimental animals may be placed in this category when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.

There has been no further evidence in humans or animals that would change this initial IARC classification. Furthermore, a more recent review of the mechanistic data indicates that the choline deficiency mechanism of action has little relevance to humans (Leung *et al.* 2005).

On March 7, 2003, OEHHA published a public notice with its decision not to proceed with the listing of DEA under the authoritative bodies mechanism (OEHHA 2003). In making such a decision, OEHHA noted that it had reviewed public comments, as well as a considerable amount of scientific information. In that notice, OEHHA concluded the following:

Because it is not clear that the scientific criteria for listing under the authoritative bodies mechanism have been met, OEHHA has decided not to proceed with the administrative listing of diethanolamine under Proposition 65.

By now considering the automatic Labor Code listing mechanism, OEHHA is overlooking its own completed Proposition 65 review of DEA (OEHHA 2003). If OEHHA proceeds with the intended Labor Code Listing, the outcome will be at odds with its own 2003 review. Other than mechanistic data, there are essentially no new carcinogenicity data on DEA since IARC's initial review (IARC 2000) and OEHHA's review (OEHHA 2003). In both the IARC and OEHHA reviews, the NTP chronic study of DEA in mice served as the key study (NTP 1999).<sup>5</sup>

When IARC's review group met in February 2011 to evaluate DEA again, IARC also had no new carcinogenicity data to review that was substantially different from the data available during the initial 2000 IARC review or OEHHA's 2003 review. However, IARC changed its conclusion for DEA to a category 2B, "possibly carcinogenic to humans" (Grosse *et al.* 2011). The IARC conclusion changed because IARC amended its own 2006 Preamble criteria for categorizing chemicals as carcinogens. This criteria change, not any new scientific evidence, was the single major factor in IARC reclassifying DEA from Group 3 (not classifiable as to its carcinogenicity to humans) to Group 2B carcinogen (possibly carcinogenic to humans).

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<sup>5</sup>NTP's findings in its chronic study with mice are not consistent with other studies conducted with DEA designed to determine its mutagenic or tumorigenic potential. In contrast to the NTP 1999 chronic mouse study, results of the NTP chronic rat study indicated no evidence of carcinogenicity (NTP 1999). DEA was not considered genotoxic in either *in vitro* or *in vivo* studies (Dean *et al.* 1985; Loveday *et al.* 1989; Hedenstedt 1978; Haworth *et al.* 1983; Inoue *et al.* 1982; Melnick *et al.*, 1988; Myhr *et al.* 1986). DEA also was negative in a short-term cancer study conducted by the National Institute of Environmental Health Sciences (NIEHS) using Tg.AC (transgenic) mice, which are bred to be very sensitive to a material's potential to cause cancer (Spalding, *et al.* 2000; Tennant *et al.* 1995).



B. IARC's Preamble Change in Carcinogenicity Criteria is Founded Neither on Sound Scientific Principals of Cancer Study Design, nor on Good Laboratory Practices

The 2006 IARC Preamble change allows for an increased incidence of tumors in both sexes of a single species to provide sufficient evidence for carcinogenicity in animals. This essentially allows results from each sex (male, female) from a single study to constitute its own independent study. Therefore, with this Preamble change, IARC re-classified the single NTP chronic mouse study of DEA as two separate positive studies.

It has been advocated previously by ACC that IARC's Preamble change is founded neither on sound scientific principles of cancer study design, nor on Good Laboratory Practices of what constitutes a study. Scientific consensus and Good Laboratory Practices support the position that a study performed at the same laboratory, in one species, in both sexes, at the same time, and with a single protocol, is properly considered as a single independent study, not as two separate evaluations, one in males and one in females (ACC 2005). The use of two species in carcinogenicity studies is based on well-founded scientific convention that no single species can be considered an adequate predictor of carcinogenic effects in humans (Gad 1995; ACC 2005). A critique of IARC's Preamble change is presented in more detail in the appended comments by ACC, which is part of this submission (ACC 2005). Therefore, the ACC Alkanolamines Panel contends that IARC's 2011 change in carcinogenicity classification of DEA was inappropriate; and by consequence, so is OEHHA's intended Labor Code listing of DEA.

C. Mechanistic Studies Provide Evidence that the Mode of Tumorigenesis of DEA in Rodents is not Relevant to Humans

The mechanistic data for DEA indicates that the mode of tumorigenesis in mice is not relevant to humans. Leung *et al.* (2005) summarized the *in vitro* and *in vivo* animal data for DEA and concluded that the experimental evidence is consistent with a mode of action involving the development of intracellular choline deficiency. Furthermore, since rodents are far more sensitive to choline deficiency than humans, the hepatocarcinogenic effect of DEA in mice is not predictive of similar susceptibility in humans (Goodman 1998). In a review of the mechanistic studies of DEA, Leung *et al.* (2005) concluded:

The fact that DEA was carcinogenic in mice but not in rats also has important implications for human risk assessment. DEA has been shown to be less readily absorbed across rat and human skin than mouse skin. Since a no observed effect level for DEA-induced choline deficiency in mice has been established to be 10 mg/kg/d, this indicates that there is a critical level of DEA that must be attained in order to affect choline homeostasis. The lack of a carcinogenic response in rats suggests that exposure to DEA did not reach this critical level. Since rodents are far more sensitive to choline deficiency than humans, it can be concluded that the hepatocarcinogenic effect of DEA in mice is not predictive of similar susceptibility in humans.

Given the mechanistic data for DEA, a Labor Code listing of DEA isn't warranted.



#### D. Limitations of the NTP Chronic Mouse Carcinogenicity Study

Also overlooked by OEHHA are the significant limitations of the NTP chronic mouse study of DEA, the basis for the 2011 IARC evaluation; and subsequently, for the planned Labor Code listing. The NTP study limitations include the following:

1. *Use of a Biologically Active Vehicle:* The NTP (NTP 1999) mouse study design used ethanol as the vehicle. Ethanol itself is not an inert material, and does not meet the criteria of a desirable vehicle (Kimmel and Francis 1990). Ethanol has been shown to promote altered cell foci and tumor formation in livers of experimental animals treated with nitrosamines (Driver and McLean 1986; Takada *et al.* 1986), and is widely regarded as a risk factor for cancer, including liver cancer (IARC 1988, Seitz and Stickel 2007). Ethanol also has been found to disrupt choline metabolism (Barak *et al.* 1973; Thompson and Reitz 1976). The same metabolic pathway(s) are believed to be involved in the formation of tumors in DEA-treated mice. The vehicle choice confounds the NTP mouse study results.
2. *Confounding of the Route of Administration:* The dermal NTP mouse study design itself was flawed in that the administration of DEA in ethanol was not occluded when applied to the skin. As such, mice would be able to lick the area and deliver a dose of DEA 30% higher than dermal alone (Stott *et al.* 2000). The amount of DEA and ethanol ingested versus the amount absorbed dermally likely varied according to dosage and even the age of the test animals; consequently, the exposures, route of administration, and dose are confounded in this study.
3. *High Incidence of Liver Tumors in Controls:* The control mice had an unusually high frequency of tumors outside most of the historical control values for NTP bioassays. The background incidence of liver tumors in control female mice and control male mice were 66% and 78%, respectively (Seilkop 1995).
4. *High Propensity for Liver Tumors in Mouse Strain:* The mouse used in the NTP chronic study was the B6C3F1 mouse. Goodman (1998) reports that the B6C3F1 mouse has a high propensity to develop liver tumors because it is deficient with regard to its ability to maintain normal DNA methylation. This observation supports the view that a mouse liver tumor response is not an appropriate endpoint for human risk assessment, particularly for agents such as DEA that are known to alter DNA methylation similar to that resulting from choline deficiency (Bachman *et al.* 2006; Goodman 1998).
5. *Use of Obese Mice:* The mice used in the study had higher body weights. Elevated body weight is, itself, a risk factor for tumor formation (Seilkop 1995).

Taken together, these study design limitations significantly confound the interpretation of results of the NTP chronic mouse study of DEA on which OEHHA's Labor Code listing ultimately rests. Consequently, these study limitations do not support a Labor Code listing for DEA.

#### E. DEA is Not Listed in NTP's Report on Carcinogens (RoC)

The single NTP chronic mouse study of DEA, the basis for the 2011 IARC classification of DEA; and now by default, the basis for the intended OEHHA Labor Code listing, was considered by three National Toxicology Program (NTP) review groups: (1) the NIEHS/NTP RoC Review Committee (or RG1) on December 10, 2001; (2) the NTP Executive Working Group for the RoC (or RG2) on May 2, 2002; and (3) the NTP Board of Scientific Counselors RoC Subcommittee on November 19-20, 2002.



Their charge was to review the NTP chronic studies of DEA (rats and mice), as well as other scientific evidence, for potential listing of DEA in NTP's RoC. All three NTP groups recommended that DEA not be listed in the RoC. All three NTP findings are summarized in the Federal Register (National Toxicology Program 2003). Consequently, DEA has never been listed by NTP in its RoC.<sup>6</sup> Other than mechanistic data suggesting that the mode of mouse tumorigenesis of DEA is not relevant for humans (Leung *et al.* 2005), no substantial data regarding the carcinogenicity of DEA has been made available since these multiple NTP decisions. Therefore, OEHHA should not proceed with its intended Labor Code decision, given that such a decision rests on the single limited NTP chronic mouse study, which NTP has already considered fully in its own decision-making process.

### **OEHHA Should Withdraw its Notice of Intent to List DEA**

OEHHA should not overlook its own previous Proposition 65 finding under the authoritative bodies mechanism not to list DEA. Nor should OEHHA overlook the findings of three NTP review bodies, namely the RG1, the RG2, and the NTP Board of Scientific Counselors ROC Subcommittee. All three NTP review groups voted against an RoC listing of DEA. OEHHA should acknowledge the significant scientific study design limitations of the NTP chronic mouse study on which it is ultimately relying. The IARC Preamble criteria change, not any new compelling scientific evidence, was the single most important factor in IARC reclassifying the carcinogenicity potential of DEA. IARC's change in its carcinogenicity classification criteria can be strongly questioned on the grounds that it does not meet sound scientific principles of cancer study design or of Good Laboratory Practices. Furthermore, evidence indicates that the mode of DEA tumorigenesis in mice is not relevant to humans. Given the mechanistic data for DEA, a Labor Code listing of DEA isn't warranted.

Because of these aforementioned considerations, the ACC Alkanolamines Panel strongly urges that OEHHA withdraw its intended listing of DEA by the Labor Code mechanism.

OEHHA has the option of reviewing DEA under its process involving the Carcinogen Identification Committee (CIC). The CIC review process is more transparent relative to the Labor Code mechanism, and allows for: (a) verbal and written input by interested parties; (b) a review in an open meeting of the CIC's proceedings; and (c) a public vote by the CIC. This more open transparent process is preferred over the "automatic" Labor Code listing, where essentially no input by interested parties is allowed. The ACC Alkanolamines Panel urges that OEHHA not only withdraw DEA from the Labor Code Listing process, but also recommends that OEHHA refer the DEA decision to the CIC.

The ACC Alkanolamines Panel has a long history, dating back to 1999, of providing scientific comment to OEHHA regarding OEHHA's various Proposition 65 reviews of DEA. The Panel thanks OEHHA for the opportunity to comment again.

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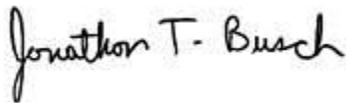
<sup>6</sup> See NTP's RoC website link: <http://ntp.niehs.nih.gov/?objectid=03C9AF75-E1BF-FF40-DBA9EC0928DF8B15>. The RoC identifies agents, substances, mixtures, and exposure circumstances that are known or reasonably anticipated to cause cancer in humans.



Should there be any questions regarding these comments from the ACC Alkanolamines Panel, I can be contacted as follows:

Jonathon T. Busch  
Manager, Alkanolamines Panel  
Director, Chemical Products and Technology Division  
American Chemistry Council  
700 2nd Street NE  
Washington DC 20002  
Office: 202 249-6725;  
Cell: 703 439-7076  
Email address: [jon\\_busch@americanchemistry.com](mailto:jon_busch@americanchemistry.com)

Sincerely,



Jonathon T. Busch



## References

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DELIVERED ELECTRONICALLY

31 October 2005

International Agency for Research on Cancer  
Lyon, France  
E-mail Address for Comment Submissions: [cie@iarc.fr](mailto:cie@iarc.fr)

Re: "Report of the Advisory Group to Recommend Updates to the IARC Monographs: Internal Report 05/001, 4-6 May 2005"

Dear Sirs,

The American Chemistry Council commends the International Agency for Research on Cancer (IARC) for soliciting widespread review by the scientific community as a whole on the draft language under consideration for revision of the IARC Preamble to the IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.<sup>1</sup> The IARC Monograph process is widely recognized for its contribution over the last 35 years to advancing environmental public health. The scientific rigor and transparency of IARC processes are critical to assuring the most up to date scientific studies and understandings are fully considered and utilized in the Monograph discussions. Opening up the proposed revisions to the Preamble to wider review is appropriate and IARC should consider even more efforts in this regard with respect to the Monographs.

The American Chemistry Council supports IARC's commitment to assessing the potential carcinogenicity of substances, and agrees that a rigorous set of scientifically robust criteria is necessary to guide the process and provide consistency across time and panels. However, we believe that two of the proposed changes do not represent an appropriate scientific approach to this process, and respectfully ask that the Advisory Group give due consideration to the comments below.

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1 The American Chemistry Council (ACC or the Council) and its member companies have played an active role in screening and testing chemical substances, developing risk assessments and implementing science-based risk management policies. ACC represents the leading companies engaged in the business of chemistry in the United States. ACC members apply the science of chemistry to make innovative products and services that make people's lives better, healthier and safer. ACC is committed to improved environmental, health and safety performance through health and environmental research and product testing, Responsible Care<sup>®</sup>, and common sense advocacy designed to address major public policy issues. Chemistry companies invest more in research and development than any other business sector. As a science-driven industry, the business of chemistry – through the Council's Long Range Research Initiative and through research, screening and testing of specific chemicals by individual member companies – provides significant support for scientific research to better understand and characterize the potential risks from chemical exposures.



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**Issue 12a: Clarify whether the National Toxicology Program (NTP) studies in male and female rats or mice should be regarded as independent studies capable of providing *sufficient evidence*.**

The issue addressed by the Advisory Group is whether an NTP study with male and female rats, for example, can be considered as two independent studies and thus be deemed “sufficient evidence” for carcinogenicity. They state: “This Advisory Group recommends that IARC update its criterion on reproducibility for sufficient evidence of cancer in experimental animals and state clearly that GLP studies in both sexes of a single species may be considered as independent.” The Council believes this position of the Advisory Group is deficient in many respects and therefore warrants further deliberation.

Although the Advisory Group is recommending in this report that such a position be adopted by IARC, there is also the following “note” provided at the beginning of the discussion of Issue 21a: “*Some Working Group members recently refused to recognize these as independent studies because they were carried out at the same time in the same laboratory using similar protocols.*”

This is a critical point and is supported by the scientific literature on cancer study design in animals: the principles of cancer study design do not support the Advisory Group’s opinion that a single study carried out in a single species, in both sexes, at the same time, in the same laboratory and under the same conditions, is actually two independent studies. The principle around which the IARC requirement for two independent studies was established is as current today as when it was initially formulated. Replication in an independent study is a cornerstone of science. Under Good Laboratory Practices (GLPs), each study is required to have a unique protocol. Therefore, if both sexes of an animal species were specified in a single protocol, this would be classified under GLP as a single study. Although GLPs provide a high degree of integrity and certainty in terms of test compound identification, test methods and procedures and documentation of laboratory measurements, GLPs do not supplant the scientific certainty provided by replication in an independent study. Even if studies of each sex of the same species were conducted under separate GLP protocols, but the studies were run simultaneously in the same laboratory using the same procedures and test article dosages, these would need to be viewed as a single study for purposes of an IARC evaluation. Because the carcinogenic process can be influenced by genetics and by exogenous factors, by impurities in test articles, by nutritional factors and by animal husbandry and the lab housing environment, not all of which can be rigorously controlled nor fully appreciated in a single study, the standard of practice which has developed has been that two independent and separate studies are needed to provide the requisite degree of scientific certainty. This standard, based on the foundation of the scientific method – independent replication - has stood the test of time and should remain a hallmark of IARC evaluations.

The scientific consensus on proper cancer study design and interpretation is widely held and is covered well in toxicology textbooks. These texts are consistent in supporting the position that a study performed at the same laboratory, in one species, in both sexes, at the same time, is properly considered as a single independent study, not as two separate evaluations, one in males and one in females. For example, when design of a carcinogenicity study is described, each study is always described as comprising rats or mice exposed for 2 years, and in each species, equal numbers of male and female animals are used.<sup>2</sup> In another

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<sup>2</sup> See, for example, Ballantyne, B. et al. 1993. *General and Applied Toxicology*, volume 2, Input Typesetting, Ltd.: Wimbledon, UK, chapter 41; Hayes, A.W. 1994. *Principles and Methods of Toxicology*, Third Edition, Raven Press: New York, chapter 19.

text discussing the safety assessment of pharmaceuticals, it is stated: “*The use of two species in carcinogenicity studies is based on the traditional wisdom that no single species can be considered an adequate predictor of carcinogenic effects in humans. Absence of carcinogenic activity in two different species is thought to provide a greater level of confidence that a compound is “safe” for humans than data derived from a single species.*”<sup>3</sup>

In another textbook discussing toxicology and safety testing of products for human use, it is stated, “definitive evidence of carcinogenicity is difficult to establish from the results of a single study”.<sup>4</sup> Therefore, whether the issue is establishing carcinogenicity or ruling out the possibility of carcinogenicity, the consensus of scientists has been that more than one study, in more than one species, not one study in both sexes, is needed. None of the standard resources on cancer study design suggest that a study in one species, both sexes, could be considered as two independent studies.

The basis for such “standards of practice” is found in the way that an NTP study is designed. The study is designed to limit variability in response among animals in the study in order to maximize the ability to detect a positive response that can be attributed to the test chemical. This is done by rigidly controlling animal husbandry (light/dark cycles, diet, temperature of environment, housing conditions, etc.) and using animals that are genetically similar, from the same species and the same breeding source. These aspects are discussed in detail in Hayes’ *Principles and Methods of Toxicology*.<sup>5</sup> By limiting such variability among animals within a single study, any identified cancer response can more likely be attributed to the test chemical rather than the well-established confounding factors in cancer study design of diet, environment, and genetics; these factors are known to be associated with increased cancer risks for certain types of cancer under certain conditions. Therefore, the study design of the NTP studies was not developed in order to produce a study that would be capable, by itself, of defining carcinogenic potential of a test substance. Rather, it was developed in order to maximize the ability to attribute a positive response to the test substance rather than some other confounding factor.

The NTP studies, however, include animals of both sexes in order to attempt to identify the contribution of gender to the response. It is well established that there are differences in the way male and female animals may respond to chemicals, including gender-related differences in carcinogenic potential<sup>6</sup> Further, as pointed out in the IARC Preamble, section 9, under “qualitative aspects,” considerations of importance to the Working Group include whether animals of both sexes were used in the study under consideration. The inclusion of animals of both sexes is a characteristic of a single study that makes it adequate for risk assessment. Then, in the same section under the heading “quantitative aspects,” it is stated that the probability that tumor will occur may depend on sex of the animal. These statements are fundamental concerns that must be considered. Sex is a characteristic that can affect response. As a result, considering a single study performed in both sexes, in a single laboratory, under the same test conditions, at the same time, does not argue for consideration of the study as two independent evaluations. In fact, with the potential for gender to affect response, if there was a positive carcinogenic response in only one species, the study would need to be repeated in order to clarify whether that response was indeed gender-related.

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3 Gad, S.C. 1995. *Safety Assessment of Pharmaceuticals*, Van Nostrand-Rheinhold: New York, chapter 7, page 168.

4 Chengelis, C.P. et al. 1995. *Regulatory Toxicology*, Raven Press: New York, chapter 3, page 66.

5 Hayes, 1994. *Principles and Methods of Toxicology, Third Edition*, Raven Press: New York, chapter 19.

6 Id.

Furthermore, if a single study in one species that included male and female animals was considered as two independent studies, and thus served as the basis of “sufficient evidence” in an IARC evaluation, an important potential factor may be overlooked: species differences in toxicological response. It is well established that both qualitative and quantitative differences in response to toxic substances may occur among different species.<sup>7</sup> Some of these species-related responses, such as the production of liver tumors after exposure to peroxisome proliferators and nasal tumor development after exposure to formaldehyde, are well documented.<sup>8</sup> It is the potential for such responses to go undetected if only one species, or one sex, was tested that has driven the “standards of practice” and the design of cancer testing requirements to include more than one study in more than one species.

Considering the scientific principles behind cancer study test design and what is known about carcinogenic responses in animals, there is no sound basis to support use of the results of a single study, in one species, in two sexes, as two independent evaluations of carcinogenic potential. Therefore, whether a study is an NTP study or not, studies run on both sexes in the same animal room, using the same species and source of animals, using the same dose solutions, and identical test conditions should be considered a single study. The certainty afforded by replication in an independent study is not fully replaced by the certainty afforded when a study is conducted under GLPs.

**Issue 5c. The 2003 Advisory Group recommended that the issues of ‘bias of opinion’ and ‘conflict of interests’ be discussed in the Preamble.**

The proposed preamble indicates that experts with real or perceived conflicts of interest will be excluded from Working Groups but can be "Invited Specialists" (page 4, lines 37-38 and line 42). Invited Specialists cannot serve as meeting chair or subgroup chair, cannot draft text that pertains to cancer data, and cannot participate in the evaluations.

As we explain below, the challenge for IARC is to assure that panels are composed of the most qualified experts, irrespective of affiliation. Affiliation alone should not be taken as synonymous with a conflict of interest. IARC can achieve the Agency’s objectives by assuring a balance across affiliations, of equally qualified experts, and by insisting that all potential conflicts be fully and transparently declared. IARC’s proposed new policy of including “specialists” in the review process does not achieve this goal. The proposed exclusion of qualified experts based solely on a perception of “commercial interests” is not justified.

Industry’s commitment to scientific research and product testing includes employing and working with the highest quality scientists. Many industry-employed scientists have national and international stature in the scientific community, and are leading experts in their disciplines. These scientists are objective, highly skilled professionals. As members of professional associations like the Society of Toxicology, industry scientists adhere to both personal and professional commitments to act in accordance with the codes of ethics of their professions.

The US National Academies’ panel selection policy emphasizes that knowledge, training and experience are the foremost considerations, and that no one should be appointed to a panel to represent a particular

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<sup>7</sup> Klaassen, C.D. 2001. *Casarett & Doull’s Toxicology: The Basic Science of Poisons*, sixth edition, chapter 2.

<sup>8</sup> Id.

point of view or special interest.<sup>9</sup> Importantly, the NAS states that “[f]or some studies . . . it may be important to have an ‘industrial’ perspective or an ‘environmental’ perspective,” not because these “sides” need to be represented, but “because such individuals, through their particular knowledge and experience, are often vital to achieving an informed, comprehensive, and authoritative understanding and analysis of the specific problems and potential solutions to be considered by the committee.”<sup>10</sup> In many if not most cases, industry scientists will be able to provide just this sort of expertise to IARC Working Groups.

As explained in a recent article addressing just this topic,<sup>11</sup> any discussion of the issue of “commercial interest” must carefully distinguish between conflict of interest and point of view. In general, true conflicts of interest are limited to instances where a person has a concrete financial interest in the subject being addressed. However, an individuals’ affiliation or point of view are not, and should not be, viewed as automatic criteria for deselection. The National Academies state: “points of view or positions that are largely intellectually motivated or that arise from the close identification or association of an individual with a point of view of a particular group.”<sup>12</sup> Similarly, an EPA Science Advisory Board committee has stated that, “[a]lthough it is possible to avoid conflict of interest, avoidance of bias is probably not possible. All scientists carry bias due, for example, to discipline, affiliation and experience”<sup>13</sup>. Fifteen past presidents of the Society of Toxicology have written in *Risk Policy Report* that, “[o]f course, all scientists have biases; acknowledging this, we as a society must be aware of those biases and seek to ensure balance in the scientific panels whose task is to provide the best possible technical review of complex, important issues.”<sup>14</sup>

In its discussion of “conflict of interest,” the National Academies’ panel selection policy refers to “financial interests,” and notes that that these can arise across the board, including regulated entities, the government and non-governmental organizations.<sup>15</sup> Importantly, NAS explains that “biases” should not be disqualifying -- even where a person works for a company with “a general business interest in” the subject of the panel -- unless the person “is totally committed to a particular point of view and unwilling, or reasonably perceived to be unwilling, to consider other perspectives or relevant evidence to the contrary.”<sup>16</sup>

As can be seen, therefore, the proposed exclusion for “commercial interests” would logically apply to anyone receiving compensation or support in any manner for their research or investigations on substances considered by IARC, to the extent that their professional livelihood could be involved. Similarly, “commercial interest” would include individuals from NGOs who are dependent on gifts and grants from institutes or foundations or individuals as compensation for work/research on a substance.

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9 “The National Academies Study Process,” available at <http://www4.nationalacademies.org/news.nsf/isbn/07302001?OpenDocument> (NAS 2001).

10 Id.

11 “Assessing the Reliability and Credibility of Industry Science and Scientists,” *Envtl. Health Persps.* doi:10.1289/ehp.8417 available via <http://dx.doi.org/> [Online 6 October 2005].

12 NAS 2001.

13 EPA Science Advisory Board Env’tl Health Cmte, letter report re “Review of the Draft Report to Congress ‘Characterization of Date Uncertainty and Variability in IRIS Assessments, Pre-Pilot vs post-Pilot,’” EPA-SAB-EHC-LTR-00-007 (Sept. 26, 2000), available at <http://www.epa.gov/sab/pdf/ehcl007.pdf>.

14 *Risk Policy Report* (Jan. 21, 2002).

15 NAS 2001.

16 Id.

The Council thus suggests that IARC review and consider adopting the National Academies' panel selection policy rather than attempting to implement the problematic, proposed approach regarding "commercial interests."

Yours sincerely,

*Original Signed By*

Richard A. Becker, Ph.D., DABT  
Senior Toxicologist and Senior Director<sup>17</sup>  
American Chemistry Council

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<sup>17</sup> Compensation Disclaimer: I am employed full-time by the American Chemistry Council, and as such was compensated for submitting these comments as part of my normal professional duties.



# Review of the carcinogenic activity of diethanolamine and evidence of choline deficiency as a plausible mode of action

Hon-Wing Leung<sup>a,\*</sup>, Lisa M. Kamendulis<sup>b</sup>, William T. Stott<sup>c</sup>

<sup>a</sup> *Independent Consultant, 15 Deer Park Road, Danbury, CT 06811, USA*

<sup>b</sup> *Indiana University School of Medicine, Indianapolis, IN 46202, USA*

<sup>c</sup> *Dow Chemical Company, Midland, MI 48640, USA*

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## Abstract

Diethanolamine (DEA) is a chemical used widely in a number of industries and is present in many consumer products. Studies by the National Toxicology Program (NTP) have indicated that lifetime dermal exposure to DEA increased the incidence and multiplicity of liver tumors in mice, but not in rats. In addition, DEA was not carcinogenic when tested in the *Tg.Ac* transgenic mouse model. Short-term genotoxicity tests have yielded negative results. In view of these apparent inconsistencies, we have critically evaluated the NTP studies and other data relevant to assessing the carcinogenic potential of DEA. The available data indicate that DEA induces mouse liver tumors by a non-genotoxic mode of action that involves its ability to cause choline deficiency. The following experimental evidence supports this hypothesis. DEA decreased the hepatic choline metabolites and *S*-adenosylmethionine levels in mice, similar to those observed in choline-deficient mice. In contrast, DEA had no effect in the rat, a species in which it was not carcinogenic at a maximum tolerated dose level. In addition, a consistent dose–effect relationship had been established between choline deficiency and carcinogenic activity since all DEA dosages that induced tumors in the NTP studies were also shown to cause choline deficiency. DEA decreased phosphatidylcholine synthesis by blocking the cellular uptake of choline *in vitro*, but these events did not occur in the presence of excess choline. Finally, DEA induced transformation in the Syrian hamster embryo cells, increased *S*-phase DNA synthesis in mouse hepatocytes, and decreased gap junctional intracellular communication in primary cultured mouse and rat hepatocytes, but all these events were prevented with choline supplementation. Since choline is an essential nutrient in mammals, this mode of action is qualitatively applicable to humans. However, there are marked species differences in susceptibility to choline deficiency, with rats and mice being far more susceptible than other mammalian species including humans. These differences are attributed to quantitative differences in the enzyme kinetics controlling choline metabolism. The fact that DEA was carcinogenic in mice but not in rats also has important implications for human risk assessment. DEA has been shown to be less readily absorbed across rat and human skin than mouse skin. Since a no observed effect level for DEA-induced choline deficiency in mice has been established to be 10 mg/kg/d, this indicates that there is a critical level of DEA that must be attained in order to affect choline homeostasis. The lack of a carcinogenic response in rats suggests that exposure to DEA did not reach this critical level. Since rodents are far more sensitive to choline deficiency than humans, it can be concluded that the hepatocarcinogenic effect of DEA in mice is not predictive of similar susceptibility in humans.

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**Keywords:** Diethanolamine; Carcinogenicity; Mode of action; Choline deficiency; Mice; Human; Risk assessment

## 1. Introduction

Diethanolamine (DEA; CASRN 111-42-2) is an alkanolamine used in a variety of industrial processes (CIR,

1983) and its fatty acid condensates are present in many consumer products (CIR, 1986). The toxicology of DEA was exhaustively reviewed by Knaak et al. (1997). However, subsequent to this review, additional data have come to light; in particular, long-term toxicity studies that suggest that DEA could have carcinogenic potential in experimental animals (NTP, 1999a). The purpose of the present re-

\* Corresponding author.

E-mail address: [hleung18@comcast.net](mailto:hleung18@comcast.net) (H.-W. Leung).

view is to critically evaluate these new studies and other data relevant to assessing the oncogenic potential of DEA. In addition, an overview of a plausible mode of action for DEA carcinogenicity and the supporting experimental evidence is presented.

### 1.1. Cancer studies in rodents (Table 1)

F-344/N rats (50/sex/group, 6 wk old) were dosed with DEA (purity >99%) in 95% ethanol by dermal application 5 d/wk for 104 wk. Males received 0, 16, 32 or 64 mg/kg body weight and females 0, 8, 16 or 32 mg/kg. Doses were selected based on results of a subchronic (13 wk) study and represented maximum tolerated doses. Survival rates for the dosed groups were similar to those of corresponding control groups. The mean body weight of the high-dose male group was lower than that of the controls from wk 8 and the mean body weight of the high-dose female group was lower than that of the controls from wk 97. No increase in tumors in the treated groups compared with the controls was observed (NTP, 1999a).

B6C3F<sub>1</sub> mice (50/sex/group, 6 wk old) were dosed with 0, 40, 80 or 160 mg/kg DEA (purity >99%) in 95% ethanol by dermal application 5 d/wk for 104 wk. Survival of dosed male mice was similar to that of the controls, but survival of dosed female mice was reduced (88, 66, 66, and 46% for the control, low-, mid-, and high-dose groups, respectively). The mean body weights of the mid- and high-dose males were lower than those of the controls after wk 88 and 77, respectively. The mean body weights of the low-

and mid-dose females were lower than those of the controls from wk 73, but those of the high-dose females were reduced compared with the controls from wk 53. In male mice, the incidences of hepatocellular adenoma and of hepatocellular adenoma and carcinoma (combined) in all dosed groups were significantly higher than those in the controls (hepatocellular adenoma: 62, 84, 98, and 90%;  $p < 0.001$ , Poly-3 trend test; hepatocellular carcinoma; 24, 34, 66, and 68%;  $p < 0.001$ , Poly-3 trend test, for the control, low-, mid-, and high-dose groups, respectively). In addition, the incidences of hepatoblastoma in the mid- and high-dose groups were significantly increased compared with the controls (0, 4, 16 ( $p = 0.004$ ), and 10% ( $p = 0.028$ ), pairwise comparisons) in the control, low-, mid-, and high-dose groups, respectively. In the female mice, the incidences of liver neoplasms were significantly higher than those in the controls (hepatocellular adenoma: 64, 100, 96, and 96%;  $p < 0.001$ , Poly-3 trend test, and hepatocellular carcinoma: 10, 38, 76, and 84%,  $p < 0.001$ , Poly-3 trend test, for the control, low-, mid-, and high-dose groups, respectively). Renal tubule adenomas in males showed a marginal increase after standard single-section examination (2, 8, 12, and 12%,  $p = 0.05$ , Poly-3 trend test, in the control, low-, mid-, and high-dose groups, respectively). When combining single with extended step-sectioning, the incidences were 2, 12, 16, and 14%,  $p = 0.055$  (NTP, 1999a).

Lower DEA dosages were selected for the cancer bioassay in rats than in mice based on results of subchronic studies, which demonstrated that rats exhibited a greater sensitivity

Table 1  
Genotoxicity data and cancer studies in laboratory animals of DEA and its fatty acid condensates

	DEA	Coconut oil acid	Lauric acid	Oleic acid
CAS registry number	111-42-2	68603-42-9	120-40-1	93-83-4
DEA content (%)	>99	19.6 <sup>a</sup>	5 <sup>a</sup>	7.3 <sup>a</sup>
<i>Genetic toxicity test</i>				
Gene mutation	Negative	Negative	Negative	Negative
Chromosomal aberration	Negative	Negative	Negative	Not tested
Sister chromatid exchange	Negative	Negative	Positive	Not tested
Micronucleus	Negative	Positive	Negative	Not tested
<i>Conventional bioassay</i>				
Rat dosage (mg/kg/d)	16, 32, 64 M 8, 16, 32 F	50, 100	50, 100	50, 100
Tumors	No increase	Kidney in F	No increase	No increase
Mouse dosage (mg/kg/d)	40, 80, 160	100, 200	100, 200	15, 30
Tumors	Liver in M&F kidney in M	Liver in M&F kidney in M	Liver in F	No increase
<i>Transgenic mouse</i>				
Dosage (mg/kg/d)	200, 400, 800	100, 200, 300	200, 400, 800	16, 32, 49
<i>Tg.Ac</i>	Negative	Negative	Positive	Negative
<i>P53<sup>+/-</sup></i>	Not tested	Negative	Negative	Negative
<i>NTP's conclusion for evidence of carcinogenicity</i>				
Male rat	No	No	No	No
Female rat	No	Equivocal	No	No
Male mouse	Clear	Clear	No	No
Female mouse	Clear	Clear	Some	No
Reference	NTP, 1999a	NTP, 1999b	NTP, 1999c	NTP, 1999d

<sup>a</sup> The DEA contents for the fatty acid DEA condensates were not analytically determined. They represented values reported by the manufacturers of the test materials.

to dermal toxicity (acanthosis and hyperkeratosis) and hematological effects (normochromic anemia) than mice.

In a bioassay employing a transgenic animal model, female *Tg.AC* mice, which carry a  $\zeta$ -globin promoted *v-Haras* transgene on a FVB background, (15–20/group, 14 wk old) were topically dosed with 5, 10, and 20 mg/mouse/application DEA in 95% ethanol 5 times/wk for 20 wk. The dosing volume was 0.2 mL/application. These doses were equivalent to about 200, 400, and 800 mg/kg body weight, respectively, and were higher than the maximum tolerated doses received by the B6C3F<sub>1</sub> mice in the conventional bioassay (NTP, 1999a). The concurrent negative control groups were similarly dosed with 0.2 mL 95% ethanol. The positive control group was administered 1.25  $\mu$ g 12-*O*-tetradecanoylphorbol 13-acetate (~99% pure) twice/wk for 20 wk. Survival was high in both the control (90%) and treated groups (80–93%). Lesions were diagnosed as papillomas when they reached at least 1 mm in diameter and persisted for 3 wk. Animals that did not survive until the end of wk 10 were excluded. All surviving mice were killed 6 wk after the last application. No evidence of chronic irritation or ulceration was observed at the site of application in all groups. In contrast to the positive controls, which developed multiple papillomas in 18 of 20 animals, no increase in the incidence of skin tumors in DEA-treated animals was seen (Spalding et al., 2000).

Cancer bioassays of three commonly used fatty acid-DEA condensates by dermal application in F-344 rats and B6C3F<sub>1</sub> mice have also been conducted by NTP (Table 1). These were coconut oil acid, lauric acid, and oleic acid condensates of DEA (NTP, 1999c,d). The same three condensates were also tested in the transgenic *Tg.AC* and *p53*<sup>+/-</sup> mouse models (Spalding et al., 2000). Details about these studies are not discussed in this review since their relevance to the evaluation of the carcinogenic potential of DEA per se has been questioned (IARC, 2000). This judgment was based on the fact that: (1) the chemical substances tested were complex mixtures of imprecise composition, (2) the actual DEA content had not been measured in any of the three studies and therefore the levels of exposure were indeterminable, and (3) these studies were not designed as, and did not represent, valid cancer bioassays of DEA. This latter point is corroborated by the observation of positive genotoxicity test results with coconut oil acid and lauric acid DEA condensates, and a positive result in the *Tg.Ac* transgenic mouse with the lauric acid DEA condensate (NTP, 1999b,c). These positive findings were contrary to those of pure DEA alone, which showed negative results from similar tests even at much higher molar dosages (NTP, 1999a).

### 1.2. Pharmacokinetics of DEA in animals

The disposition of DEA varies across species, is dosage-dependent, and is characterized by a relatively long elimination (Mathews et al., 1995; Mendrala et al., 2001). Absorption of DEA applied dermally in rats varies with dosage, ranging from 3 to 16% in 48 h at dosages of 2

and 28 mg/kg, respectively (Mathews et al., 1997). In mice, doses ranging from 8 to 80 mg/kg were more readily absorbed through the skin (25–60% in 48 h). Skin penetration rates determined in vitro using full-thickness skin preparations also confirmed that dermal penetration of DEA in mice was much higher than that in rats (46.3 and 1.8  $\mu$ g/cm<sup>2</sup>/h, respectively) (Sun et al., 1996). Once absorbed, DEA displays a biphasic clearance from the blood, with a rapid initial phase (half-life of ~0.1 h) followed by a prolonged phase (half-life of ~7 d) (Mendrala et al., 2001) consistent with its accumulation in the liver and kidney. The long elimination half-life is attributed to incorporation of DEA into phospholipids (Mathews et al., 1995), which may lead to bioaccumulation with chronic dosing.

### 1.3. Genotoxicity data

DEA has been evaluated for genotoxic activity in a broad array of in vitro and in vivo assays. The results of these tests have been consistently negative, indicating a lack of genotoxicity. DEA was not mutagenic to *Salmonella typhimurium* strains TA100, TA1535, TA1538 or TA98 (Haworth et al., 1983; Dean et al., 1985; NTP, 1999a), or to *Escherichia coli* WP2 uvrA (Dean et al., 1985), in the presence or absence of metabolic activation. No induction of mutation in mouse lymphoma L5178Y cells at the Tk locus (Myhr et al., 1986) or mitotic gene conversion in JD1 strain of *Saccharomyces cerevisiae* (Dean et al., 1985) was observed. Also, no induction of sister chromatid exchange in Chinese hamster ovary (CHO) cells was seen (Sorsa et al., 1988; Loveday et al., 1989). Furthermore, no induction of chromosomal aberrations in CHO cells (Loveday et al., 1989) or rat liver cells (Dean et al., 1985), and no induction of micronuclei in the peripheral erythrocytes of DEA-treated mice (NTP, 1999a) or the larvae of the newt *Pleurodeles waltl* exposed to DEA in vivo (Fernandez et al., 1993) was observed.

### 1.4. Cancer epidemiology studies in humans

No studies were identified that examined the risk of cancer among persons exposed exclusively to DEA (IARC, 2000). However, DEA has been used as an additive for metalworking fluids and as wetting fluids for asphalt paving. Road paving and roofing materials are complex mixtures containing many known or suspected carcinogens such as benzene, 1,3-butadiene and coal tar pitches. Metalworking fluids are also complex mixtures that may vary considerably depending on the type of fluid and the additives used. Studies of asphalt and road-maintenance workers have been reviewed (Partanen and Boffetta, 1994), and studies of workers exposed to metalworking fluids have been reviewed recently (IARC, 2000). IARC concluded that these epidemiological studies were not informative for the evaluation of carcinogenicity of DEA, because of the unknown probability of exposure and the potential for confounding from mixed exposure to other known or suspected animal carcinogens.

### 1.5. Conclusions regarding evidence of carcinogenicity

The National Toxicology Program's evaluation of the 2-year dermal studies for DEA (NTP, 1999a) concluded that there was "no evidence" of carcinogenic activity in male or female F344/N rats, and "clear evidence" of carcinogenic activity in male and female B6C3F<sub>1</sub> mice based on increased incidences of liver neoplasms in males and females and increased incidences of renal tubule neoplasms in males. Before a final determination as to whether DEA should be listed as a carcinogen in the Report on Carcinogens, three scientific review panels, two composed of federal scientists and one of non-governmental scientists, were tasked to evaluate all the information relevant to the inquiry. All three panels concluded that DEA did not meet NTP's criteria for listing as a carcinogen because of insufficient rodent cancer data (Federal Register, 2003). Similarly, IARC has concluded that there is inadequate evidence in humans and limited evidence in experimental animals for the carcinogenicity of DEA. IARC's overall evaluation of DEA is Group 3, i.e., not classifiable as to its carcinogenicity to humans (IARC, 2000).

### 1.6. Potential mode(s) of action

Because all the mutagenicity and clastogenicity studies for DEA were negative (Table 1), a non-genotoxic mode of tumorigenic action is indicated. Non-genotoxic chemicals may cause liver tumors in mice through a variety of mechanisms (Goodman et al., 1991). Three potential modes of action have been hypothesized for DEA: (1) perturbation of choline homeostasis; (2) perturbation of phospholipid metabolism; (3) in situ formation of *N*-nitrosodiethanolamine. As we show in later sections, the available experimental evidence indicate that the most plausible explanation for the carcinogenic effects for DEA concerns its ability to alter choline homeostasis leading to biochemical conditions consistent with the development of a chronic choline deficiency induced hepatocarcinogenesis (Fig. 1).

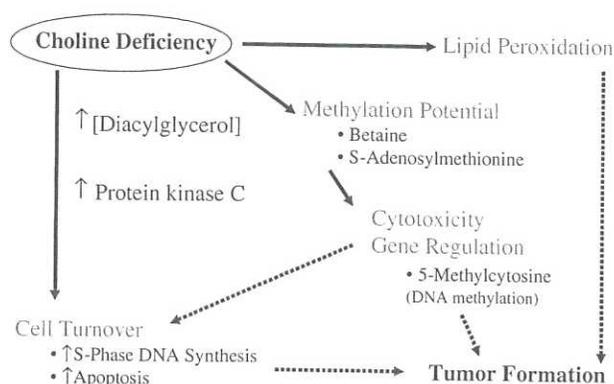


Fig. 1. Biological events involved in choline deficiency as a mode of action for inducing tumor formation.

### 2. Effects of DEA on choline homeostasis

Choline is an essential nutrient in all mammals, and dietary choline deficiency is known to promote spontaneous hepatocarcinogenesis in rodents (Newberne et al., 1982; DeCarmargo et al., 1985; Rogers et al., 1987). The principal source of choline in mammalian species is via their diet. In the liver, the primary amino group of the ethanolamine portion of phosphatidylethanolamine (PE) is methylated to form phosphatidylcholine (PC). Methylation of PE to PC followed by phospholipase cleavage of PC to choline and 1,2-*sn*-diacylglycerol (DAG) represents the only pathway of de novo choline synthesis in adult mammals (Fig. 2). The presence of *N*-methyl and *N,N*-dimethyl DEA in the serum of exposed rats as well as the presence of methylated DEA head groups in liver phospholipids (Mathews et al., 1995) is an indication that phosphatidyl DEA is methylated and cleaved by the same pathway.

DEA is a competitive inhibitor of choline and a mixed inhibitor of ethanolamine incorporation into PC and PE (Barbee and Hartung, 1979a). In male Sprague–Dawley rats, a single dose of 250 mg/kg DEA had no effect on phospholipid biosynthesis, but repeated daily dosing reduced ethanolamine incorporation into liver phospholipids to 27% of control after 1 wk, while choline incorporation fell to 41% of control after 3 wk. These results suggest that animals receiving sustained exposure to DEA over a long period of time could become choline deficient. Since the disposition of DEA favors accumulation with chronic administration, the development of choline deficiency would be progressive, increasing in severity with time.

When animals are fed a choline-devoid diet, an acute choline deficiency develops and significantly reduces the synthesis of phospholipid. Since PC is a major constituent of lipoprotein envelopes, the inability to form these structures inhibits the secretion of triglyceride and leads to the accumulation of fat in the liver. Indeed, the histopathological hallmark of choline deficiency is hepatic fatty metamorphosis. Choline deficiency is known to cause accumulation of DAG in the liver. This results in a stable activation of protein kinase C (PKC) and/or an increase in the total PKC pool in the cell with changes in several PKC isoforms (daCosta et al., 1993). Abnormalities in PKC-mediated signal transduction may be the trigger for carcinogenesis (Weinstein, 1990) and chronic stimulation of PKC has been implicated in enhanced cell proliferation (Blusztajn and Zeisel, 1989). Therefore, in addition to altering lipid membrane composition and triglyceride transport, choline deficiency is also associated with various processes that increased sensitivity to hepatocarcinogenesis.

In the liver, choline is taken up by active transport and rapidly phosphorylated to phosphocholine, which commits it to be utilized for PC synthesis (Fig. 2). In rodents, the biochemical hallmark of dietary choline deficiency is the depletion of cellular phosphocholine, which is reduced by nearly 80% (Pomfret et al., 1990). Choline that is not phosphorylated is oxidized to betaine, which serves as a methyl

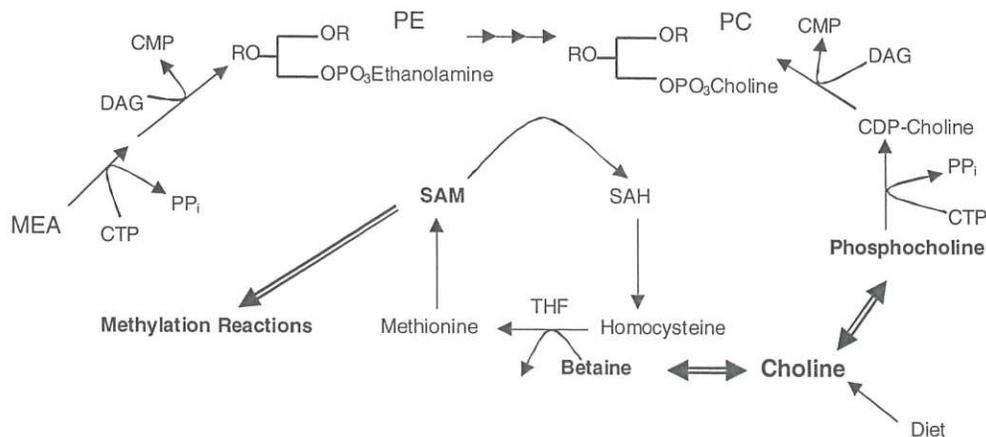


Fig. 2. Inter-relationship between the intracellular pathways for the utilization of choline and methionine. Choline is utilized in phosphatidylcholine biosynthesis or oxidized to betaine, which serves as the methyl donor in the conversion of homocysteine to methionine. In this manner, the generation of methionine from homocysteine intersects choline and 1-carbon metabolic pathways. Methionine, as *S*-adenosylmethionine, is also an important precursor for the conversion of phosphatidylethanolamine to phosphatidylcholine, a pathway that is most active in liver. The parts of pathways particularly perturbed by DEA are highlighted in bold markings. CDP-choline: Cytidyl diphosphate-choline; CMP: cytidyl monophosphate; CTP: cytidyl triphosphate; DAG: diacylglycerol; MEA: monoethanolamine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PP<sub>i</sub>: pyrophosphate; SAH: *S*-adenosylhomocysteine; SAM: *S*-adenosylmethionine; THF: tetrahydrofolate.

donor in the regeneration of methionine from homocysteine. Methionine is subsequently converted to *S*-adenosylmethionine (SAM), the active methylating agent for many enzymatic reactions (Zeisel and Blusztajn, 1994). This reaction establishes a pathway between the methyl groups of choline and the 1-carbon pool (Fig. 2). The interaction between these pathways is demonstrated by the fact that dietary choline deficiency in rats not only depletes hepatic choline and choline metabolites, but also decreases SAM (Zeisel et al., 1989). If methylated DEA cannot donate methyl groups for this reaction, then essential methyl groups are removed from the 1-carbon pool. This has the potential to reduce the availability of SAM, the source of methyl groups for the methyltransferases that methylate DNA. Hypomethylation of DNA has been shown to occur in as little as 7 d following administration of a choline-deficient diet (Wainfain et al., 1989). More importantly, hypomethylation of critical target genes as a result of reduced availability of SAM could be a critical factor in the carcinogenic response observed in mice. In fact, altered DNA methylation has been implicated in aberrant expression of genes and has been suggested as an epigenetic mechanism of carcinogenesis (Eden et al., 2003; Goodman and Watson, 2002).

Other effects associated with choline deficiency include increased generation of free radicals, increased susceptibility to oxidative damage (Rushmore et al., 1984), increased cell death (Ghoshal et al., 1983), and increased cellular proliferation (Counts et al., 1996). These changes are frequently associated with increased susceptibility to tumorigenesis.

### 3. Effects of DEA on phospholipid metabolism

Since DEA is structurally similar to choline and ethanolamine, both of which are precursors for phospholipids bio-

synthesis, it has been hypothesized that DEA may induce tumors by disrupting phospholipid metabolism, which may perturb cell and organelle membrane function, and the synthesis of fatty acid second messengers (NTP, 1999a). DEA is metabolized by biosynthetic pathways similar to those for ethanolamine. It has been shown to undergo *O*-phosphorylation, *N*-methylation, and incorporation into phosphoglyceride and spingomyelin analogs as the parent compound and as *N*-methyl and *N,N*-dimethyl derivatives. Because of its structural similarity to choline and ethanolamine, DEA can compete with these endogenous precursors in the synthesis of phospholipids (Chojnacki and Korzybski, 1963; Pelech and Vance, 1984). Choline and ethanolamine are essential in lipid metabolism and membrane homeostasis (Fleischer and Rouser, 1965; Fleischer et al., 1967). DEA can be incorporated into phospholipids (Barbee and Hartung, 1979a,b) and inhibit the *in vitro* and *in vivo* synthesis of phospholipid derivatives of choline and ethanolamine (Barbee and Hartung, 1979a; Lehman-McKeeman and Gamsky, 1999, 2000).

DEA is incorporated into phospholipid head groups by the same biosynthetic pathways as ethanolamine, leading to the formation of DEA-containing phospholipid and can alter the structure and properties of membranes containing these aberrant phospholipids. For example, DEA treatment has been shown to alter mitochondrial membrane integrity (Barbee and Hartung, 1979a) and inhibit microsomal enzyme activity (Barbee and Hartung, 1979b). These adverse effects do not occur *in vitro*, suggesting that the *in vivo* incorporation into the lipid bilayer of membranes is a prerequisite to these changes. Furthermore, the mitochondrial and microsomal effects were time-dependent, as these changes were not observed with acute treatment and increased in severity with repeated dosing (Barbee and Hartung, 1979a,b).

DEA incorporation into phospholipids may also affect the generation of lipid second messengers from DEA-containing phospholipids. Ceramide is a second messenger generated by the action of sphingomyelinase, which hydrolyzes phosphocholine from sphingomyelin to yield free phosphocholine and ceramide. Ceramide is an important component of intracellular signal transduction and differentiation (Speigel and Merrill, 1996; Speigel et al., 1996). It has been demonstrated that in DEA-treated rats, 93% of the DEA incorporated into liver phospholipids was present as ceramide derivatives, where DEA and/or phospho-DEA was incorporated into sphingomyelin in place of phosphocholine (Mathews et al., 1995). Thus, the presence of DEA-containing phospholipids in the phospholipid signaling pool would affect the ability of cells to respond to activation of the sphingomyelin pathway. Furthermore, this could also result in perturbation of intercellular communication and intracellular signal transduction. Inhibition of gap junction intercellular communication (GJIC) is a common feature of non-genotoxic carcinogens and has been suggested as a possible mechanism of action for the tumor promotion process (Yamasaki, 1990). Inhibition of GJIC was recently demonstrated in cultured rat and mouse hepatocytes treated with DEA *in vitro* (Kamendulis et al., 2004). While DEA can be incorporated into phospholipids (Artom et al., 1958; Mathews et al., 1995), experimental evidence indicates that choline deficiency and not DEA-containing phospholipids was responsible for this effect. Kamendulis et al. (2004) observed that choline supplementation eliminated loss of GJIC and that choline deficient media caused the same effect (i.e., in the absence of DEA and its metabolites). Significantly, no effect upon GJIC was noted in cultured human hepatocytes despite the fact that Mathews et al. (1995) demonstrated that metabolic incorporation of DEA into phospholipids could occur in human liver tissue. Also, since there are no known qualitative or quantitative differences in DEA incorporation into phospholipids in rats or mice, but only mice developed tumors, this suggests that perturbation of phospholipid metabolism is an unlikely mode of carcinogenic action. Furthermore, recent data suggest that choline deficiency, not incorporation of DEA into phospholipids, stimulates the induction of DNA synthesis (Kamendulis and Klaunig, 2005).

### 3.1. *In situ* formation of *N*-nitrosodiethanolamine

As a secondary amine, DEA may react with a nitrosating agent under appropriate conditions to form the nitrosamine *N*-nitrosodiethanolamine (NDELA). NDELA is mutagenic *in vitro* and reportedly causes liver tumors in rats at  $\geq 2$  mg/kg/d (ECETOC, 1990). Formation of NDELA *in vivo* has been measured or inferred in rats administered high, often toxic, oral bolus doses of both DEA and nitrite (Preussmann et al., 1981; Yamamoto et al., 1995). However, recent studies using dosing conditions mimicking those in the NTP bioassay (160 mg/kg

DEA dermally and drinking water supplemented with 170 ppm sodium nitrite to favor nitrosation) failed to detect NDELA in the gastric ingesta, blood or urine of mice given repeated carcinogenic dosages of 160 mg/kg/d (Stott et al., 2000b). This suggests that the mouse liver tumors observed in the NTP bioassay were unlikely due to *in situ* nitrosamine formation.

### 3.2. *Experimental evidence supporting choline deficiency as a mode of action*

The effect of DEA on choline homeostasis was evaluated in several independent studies. Lehman-McKeeman (Lehman-McKeeman, 2001; Lehman-McKeeman et al., 2002) reported that male B6C3F<sub>1</sub> mice dosed dermally with 160 mg/kg/d, 5 d/wk for 2 wk showed a marked decrease in choline metabolites and SAM levels in their livers similar to animals kept on a choline-devoid diet, indicating the development of choline deficiency (Table 2). These effects were reversed following a 2-wk recovery period. Stott et al. (2000a,b) have also reported significant reductions in the hepatic levels of choline metabolites, including choline, phosphocholine, and glycerophosphocholine, and SAM levels in a separate study with B6C3F<sub>1</sub> mice dosed in a similar regimen with DEA via dermal and/or oral routes. Interestingly, Lehman-McKeeman et al. (2002) observed that the C57Bl/6 mouse strain, which has a low spontaneous liver tumor incidence compared with the B6C3F<sub>1</sub>, when similarly dosed with DEA, showed a decrease in choline metabolite levels, but no change in hepatic SAM levels. In contrast, in male F-344 rats, DEA did not alter the hepatic concentrations of choline metabolites or SAM at the maximum dosage of 64 mg/kg used in the NTP bioassay (Lehman-McKeeman, 2001; Lehman-McKeeman et al., 2002). These data support a causal role for choline depletion in the development of mouse liver neoplasm by DEA.

To further examine the choline deficiency hypothesis, a comprehensive evaluation of dose–response relationship for changes in choline metabolite levels was conducted (Lehman-McKeeman et al., 2002). When male B6C3F<sub>1</sub> mice were dermally dosed with DEA for 4 wk, choline metabolite levels decreased in a dose-dependent manner. A no effect level was observed at 10 mg/kg (Table 3). In addition, Stott et al. (2000b) showed that the hepatic choline and phosphocholine levels in B6C3F<sub>1</sub> mice decreased in proportion to increased blood DEA concentrations (Table 4).

*In vitro* studies provided corroborative evidence supporting choline deficiency as a mode of action. In cultured CHO cells, DEA inhibited the uptake of choline, with 95% inhibition observed at 250  $\mu$ g/mL. In addition, PC synthesis was decreased at DEA concentrations  $\geq 50$   $\mu$ g/mL, while there was no effect on cell number or total phospholipid content (Lehman-McKeeman and Gamsky, 1999). Inhibition of choline uptake and decreased PC synthesis were reversed by removing DEA from the culture medium

Table 2  
Effects of DEA treatment on hepatic concentrations of choline metabolites and S-adenosylmethionine in male mice and rats<sup>a</sup>

	Phosphocholine <sup>b</sup>		Choline <sup>b</sup>		S-Adenosylmethionine <sup>b</sup>		
	Dermal	Oral	Dermal	Oral	Dermal	Oral <sup>c</sup>	Oral <sup>c</sup>
<b>B6C3F<sub>1</sub> mice</b>							
Control	1220 ± 44	1340 ± 161	155 ± 14	412 ± 88	69 ± 4	74 ± 4	96 ± 10
Choline-deficient <sup>d</sup>	297 ± 53*		95 ± 18*		50 ± 2*		
DEA <sup>e</sup>	615 ± 37*	210 ± 82*	106 ± 13*	211 ± 40*	48 ± 2*	65 ± 4*	72 ± 6*
DEA <sup>f</sup>	1224 ± 114		180 ± 21		68 ± 7		
<b>C57Bl/6 mice</b>							
Control	1166 ± 93		164 ± 19		84 ± 5		
DEA <sup>e</sup>	593 ± 69*		80 ± 7*		82 ± 2		
<b>Fischer 344 rats</b>							
Control	1547 ± 222		135 ± 16		57 ± 4		
DEA <sup>g</sup>	1286 ± 25		108 ± 14		57 ± 4		

<sup>a</sup> Data from Lehman-McKeeman (2001), Lehman-McKeeman (2002), and Stott et al. (2000a,b).

<sup>b</sup> Concentrations are presented as nmol/g liver and represent the mean ± SE of at least five rats or mice.

<sup>c</sup> Two separate experiments were performed.

<sup>d</sup> Mice were allowed ad libitum access to a choline-devoid diet for a 2-wk period.

<sup>e</sup> Mice were dosed with 160 mg/kg/d of DEA in 95% ethanol, 5 d/wk for 2 weeks.

<sup>f</sup> Mice were dosed with 160 mg/kg/d, 5 d/wk for 4 weeks and allowed a 2-wk recovery period prior to analysis.

<sup>g</sup> Rats were dosed with 64 mg/kg/d, 5 d/wk for 2 weeks.

\* Significantly different from control ( $p < 0.05$ ).

Table 3  
Levels of hepatic choline metabolites in B6C3F<sub>1</sub> mice dosed dermally with diethanolamine<sup>a</sup>

	DEA (mg/kg/d) <sup>b</sup>					
	0	10	20	40	80	160
Phosphocholine (nmol/g liver)	1220 ± 44	1192 ± 77	994 ± 78*	959 ± 70*	831 ± 52*	615 ± 37*
Glycerophosphocholine (nmol/g liver)	372 ± 34	463 ± 34	303 ± 32	275 ± 14*	281 ± 23*	193 ± 20*
Choline (nmol/g liver)	155 ± 14	137 ± 12	152 ± 11	126 ± 7	94 ± 14*	106 ± 13*
Phosphatidylcholine (μmol/g liver)	19.6 ± 1.0	17.7 ± 1.0	17.0 ± 0.4	18.0 ± 1.0	17.4 ± 0.8	16.8 ± 0.4*
S-Adenosylmethionine (nmol/g liver)	83.9 ± 1.1	84.1 ± 4.1	84.7 ± 3.9	82.1 ± 7.2	65.1 ± 6.4*	53.3 ± 4.3*
S-Adenosylhomocysteine (nmol/g liver)	48.1 ± 1.9	52.2 ± 6.2	49.1 ± 1.8	52.6 ± 1.4	61.8 ± 4.4*	58.5 ± 2.7*

<sup>a</sup> Data from Lehman-McKeeman et al. (2002).

<sup>b</sup> Mice were dosed dermally with DEA in 95% ethanol for 4 wk. Control mice were dosed with 95% ethanol. Results represent the mean ± SE of 6 mice/group.

\* Significantly different from control ( $p < 0.05$ ).

Table 4  
Levels of hepatic choline metabolites in male B6C3F<sub>1</sub> mice dosed with diethanolamine<sup>a</sup>

	Blood DEA conc. <sup>b</sup> (μg/mL)	Choline (nmol/g liver)	Phosphocholine (nmol/g liver)
Control <sup>c</sup>	0.011	412 ± 88	1340 ± 162
Dermal <sup>c,d</sup>	5.0 ± 0.8*	211 ± 40*	413 ± 49*
Dermal and oral <sup>c,e</sup>	6.6 ± 1.3*	171 ± 28*	272 ± 103*
Oral gavage <sup>e</sup>	7.7 ± 1.3*	148 ± 35*	210 ± 82*

<sup>a</sup> Data from Stott et al. (2000b).

<sup>b</sup> Determined 1–2 h after the last dose.

<sup>c</sup> Mice were dosed with 160 mg/kg/d DEA in 95% ethanol for 14 consecutive days. Control mice were dosed with 95% ethanol. Results represent the mean ± SD of 5 mice/group.

<sup>d</sup> Mice were fitted with a collar to restrict oral access to the dermal application site.

<sup>e</sup> Mice had free access to the dermal application site during normal grooming activities.

\* Significantly different from control ( $p < 0.05$ ).

and were prevented by culturing cells in the presence of excess choline (30 mM). In a study with Syrian hamster embryo (SHE) cells, DEA was found to similarly inhibit choline uptake at concentrations  $\geq 50$  μg/mL, reaching a maximum of 80% inhibition at 250–500 μg/mL (Lehman-McKeeman and Gamsky, 2000). DEA also decreased PC synthesis at concentrations  $\geq 100$  μg/mL, reaching a maximum reduction of 60% at 500 μg/mL. PC synthesis, likewise, was unaffected when DEA-treated cells were cultured with excess choline. When DEA was incubated with SHE cells, it was incorporated into phospholipids, and this process was inhibited by choline supplementation. In a companion study of morphological transformation in the SHE cell assay, exposure to DEA (10–500 μg/mL) for 7 d showed a positive concentration-related response that was abolished by co-administration with 30 mM choline (Lehman-McKeeman and Gamsky, 2000).

S-phase DNA synthesis was increased 1.8–3.2-fold over control in mouse hepatocytes following treatment with  $\geq 10$  mg/L DEA (Kamendulis and Klaunig, 2005). Incubation of mouse hepatocytes in medium containing reduced choline concentrations (1/10–1/100 of normal medium; 0.898–0.0898 mg/L) for 24 h resulted in a 1.4–2.4-fold increase in DNA synthesis over control; whereas incubation in medium containing 10-fold higher choline concentration produced a 50% reduction in DNA synthesis from control. In another study to examine the effects across different animal species, increased DNA synthesis was observed in mouse and rat, but not human hepatocytes following treatment with  $\geq 10$  mg/L DEA (Kamendulis and Klaunig, 2005). Incubation of hepatocytes in medium containing reduced choline increased DNA synthesis 1.6- and 1.8-fold of control in mouse and rat hepatocytes, respectively, but no increase was observed in human hepatocytes. Mouse and rat hepatocytes co-treated with DEA in medium supplemented with 2–50-fold excess choline reduced DEA-induced DNA synthesis to control levels or below. These results indicate that the induction of *in vitro* DNA synthesis by DEA in the liver is associated with choline depletion, and is species specific.

The induction of cell proliferation by DEA *in vivo* was studied by evaluating S-phase DNA synthesis in the liver and kidney of B6C3F<sub>1</sub> mice dosed dermally with 160 mg/kg/d DEA in 95% ethanol after 1, 4, and 13 wk (Mellert et al., 2004). DNA synthesis was increased 3.2, 2.5, and 2.2-fold after 1, 4, and 13 wk, respectively, in the centrilobular area of the liver, coincident with increases in the number of mitotic figures found. Apoptosis was increased by 30% after 13 wk of dosing. Histopathological examination of the liver revealed syncytialization in 4 of 10 DEA-treated mice after 13 wk of dosing. No increase in cell proliferation was observed in the peri-portal or intermediate areas, thus the centrilobular area appeared to be the target zone. In the kidney, enhanced DNA synthesis was restricted to the proximal tubules in the cortex and in the outer stripe of the outer medulla. Mitotic index was increased, but no increase in apoptosis or histopathological changes were observed at any time points. In mice dosed with DEA for 1 wk and then allowed 3 wk for recovery, the labeling indices in both the liver and kidney were lower than or close to the corresponding control values, indicating that the proliferative effect was reversible. Demonstration of reversibility of effects further supports the non-genotoxic mode of action for DEA. In another study where male mice were dosed with 10, 20, 40, 80, 160, 630, and 1250 mg/kg/d DEA for 1 and 13 wk, increased cell proliferation was also observed in the livers of all DEA-treated groups (Mellert et al., 2004).

Stott et al. (2000a) reported that the levels of hepatic 1,2-*sn*-diacylglycerol, a potent agonist of phosphokinase C activity, were elevated in mice dosed with DEA via oral gavage for 4 wk. While the net PKC enzyme activity was not increased, the levels of PKC  $\alpha$  and  $\delta$  isoforms were increased 2-fold. These data are consistent with enhanced cell

proliferation as a probable mode of tumorigenic action for DEA, since chronic stimulation of PKC activity by increased DAG in choline deficiency has been known to be associated with induction of cell proliferation and subsequent liver tumor formation (Blusztajn and Zeisel, 1989).

Recent studies of DNA methylation of DEA-treated mouse hepatocytes have revealed a significant decrease in 5-methylcytosine content of GC-rich promotor regions nearly identical to those found in choline deficiency (Goodman et al., 2005). Similar changes have been associated with altered gene expression and tumor formation and have also been noted in the liver of choline-deficient (Locher et al., 1986) and lipotrope-deficient (Wainfain et al., 1989) rats.

#### 4. Discussion

In lifetime dermal exposure studies, DEA increased the incidence and multiplicity of liver tumors in B6C3F<sub>1</sub> mice (NTP, 1999a). However, DEA was not carcinogenic in F-344 rats, and no increase in the incidence of skin tumors was observed in the *Tg.AC* transgenic mice. Furthermore, there was no evidence of genotoxicity in a battery of standard tests. These findings indicate that DEA induced liver tumors in mice by an epigenetic mode of action. Among the many mechanisms known to promote liver tumors in mice, the most likely one involves the ability of DEA to alter choline homeostasis leading to biochemical conditions consistent with the development of a chronic progressive choline deficiency. The development of intracellular choline deficiency as the mode of action by which DEA caused the mouse liver tumors observed in the NTP bioassay (NTP, 1999a) is supported by the following experimental evidence:

1. DEA decreased hepatic choline metabolites and SAM levels in B6C3F<sub>1</sub> mice. This pattern of changes was similar to those observed in choline-deficient mice (Lehman-McKeeman, 2001; Lehman-McKeeman et al., 2002; Stott et al., 2000a). It is remarkable that these changes occurred in mice consuming a choline-replete diet. Thus, it is likely that the actions of DEA limit intracellular availability of choline in a fashion that results in a choline-deficient condition even when choline is available in the diet.
2. Species concordance between choline deficiency and carcinogenicity was observed. In contrast to the mouse, DEA did not alter the hepatic concentrations of choline metabolites or SAM in the rat, a species in which it was not carcinogenic at a maximum tolerated dose level (Lehman-McKeeman, 2001).
3. A dose-response association has been established for DEA-induced choline deficiency and carcinogenic activity; all carcinogenic dosages of DEA; i.e., 40, 80, and 160 mg/kg used in the NTP bioassay (NTP, 1999a) caused choline deficiency (Lehman-McKeeman et al., 2002).

4. B6C3F<sub>1</sub> mice have a much lower ability than C57Bl/6 mice to maintain nascent methylation capacity, a characteristic that is believed to contribute to their sensitivity to hepatocarcinogenesis (Counts et al., 1996). When C57Bl/6 mice were treated with DEA, choline metabolites decreased, but hepatic SAM levels were unchanged (Lehman-McKeeman, 2001). These data suggest that the B6C3F<sub>1</sub> strain of mice used in the NTP bioassay (NTP, 1999a) was uniquely susceptible to the effects of DEA.
5. DEA decreased PC synthesis by blocking the uptake of choline into CHO and SHE cells. DEA itself was utilized in the PC synthetic pathway, becoming incorporated as a phospholipid head group. However, both of these events were prevented in the presence of excess choline (Lehman-McKeeman and Gamsky, 1999).
6. GJIC was inhibited by DEA as a result of choline deficiency, not the subsequent incorporation of DEA into phospholipids (Kamendulis et al., 2004).
7. DEA induced morphological transformation in SHE cells. However, in the presence of excess choline, DEA caused no morphological transformation (Lehman-McKeeman and Gamsky, 1999).
8. DNA synthesis was increased in mouse hepatocytes incubated either with DEA or in medium containing reduced choline; whereas choline supplementation prevented DEA-induced DNA synthesis (Kamendulis and Klaunig, 2005).
9. DNA hypomethylation in GC-rich promotor regions observed in primary mouse hepatocytes which have been treated with DEA are similar to those caused by choline-deficient medium (Goodman et al., 2005).

As noted earlier, the histopathological hallmark of dietary choline deficiency is fatty liver. Although DEA treatment caused biochemical changes consistent with choline deficiency, it did not cause fatty liver (Lehman-McKeeman et al., 2002). Liver weights were increased in both rats and mice after 13 wk of exposure to DEA (Melnick et al., 1994a,b), but no evidence of fat accumulation in the livers of rats or mice was seen. This would suggest an inconsistency with the proposed mode of action. However, markedly disorganized rough endoplasmic reticulum and fatty droplets have been observed in rats dosed with 1000 mg/kg DEA for 5 d (Hruban et al., 1965). In addition, mice receiving 1100–5500 mg DEA/kg body weight were reported to have vacuolization and fatty changes in the liver 4 h after intraperitoneal injection, but by 24 h, the vacuolization had disappeared and only a moderate increase in the number of fatty droplets was identifiable (Blum et al., 1972). The apparent discrepancy between acute high dose versus chronic low dose of DEA treatment could be explained by several factors. First, since DEA is incorporated into phospholipids, fatty liver may not develop if the DEA-containing lipids can function in triglyceride secretion. Second, the lack of a fatty liver may be explained by the slow, progressive development of choline deficiency in DEA-treated

animals as opposed to the abrupt removal of choline from the diet. This distinction is further supported by the observation that marginal choline deficiency (0.25% choline in diet) results in only a slight increase in fatty liver and enlarged mitochondria (Butler and Neal, 1973). Furthermore, it is well documented that the mouse model of chronic choline deficiency differs from rats because most mouse strains do not develop liver cirrhosis and hemorrhagic necrosis that is typical of chronic choline deficiency in rats (DeCarmago et al., 1985). It is also generally recognized that susceptibility to develop fatty liver in choline deficiency is age-dependent. The majority of studies examining choline deficiency began dietary modulation in weanling animals, which are highly susceptible to choline deficiency (Rogers et al., 1987). Susceptibility to choline deficiency declines rapidly, and young adults (10–12 weeks of age) are unlikely to develop features of fully expressed choline deficiency. Secondly, dietary fat composition also contributes to the development of fatty liver in choline deficiency. Specifically, the fat composition of choline deficient diets is often augmented to at least 20%, whereas standard laboratory chows contain about 5% fat. Additionally, variations in fatty liver development are observed when the fat is derived from animal or plant origins (Rogers et al., 1987; DeCarmago et al., 1985). Therefore, the lack of fatty liver in all studies conducted with DEA is likely not an inconsistency, but rather a result of the variations in the experimental conditions.

In the NTP chronic bioassay (NTP, 1999a), evidence of carcinogenicity was observed in mice but not in rats. The difference in tumorigenic response might be explained in part by absorption kinetics. Since DEA was more readily absorbed through the skin of mice than rats (Sun et al., 1996), and given that the dosages employed in the NTP bioassay were higher in mice than rats (40–160 and 8–64 mg/kg, respectively), it follows that the total exposure to DEA was markedly different between these two species. Since absorption of DEA across human skin is even less than rats (Sun et al., 1996), the levels required to alter choline homeostasis are not likely to be achieved following human exposure to DEA.

In addition, in the NTP chronic bioassay (NTP, 1999a), ethanol was used as a vehicle for the dermal dosing of DEA, with a dosage reaching as much as 1500 mg ethanol/kg/d. Bioavailability studies replicating the same dosing conditions as the NTP chronic bioassay demonstrate that such a dosage of ethanol would give rise to an ethanol body burden of about 50–62 mg/kg/d (Leibold and van Ravenzwaay, 2003). Chronic ethanol ingestion has been shown to increase hepatic choline requirements (Barak et al., 1973), and the major manifestation is a reduction in hepatic betaine levels (Chern et al., 2000). As illustrated in Fig. 1, betaine, the oxidation product of choline, is an important 1-carbon donor utilized in the maintenance of methyl group donor levels (i.e., SAM). Lehman-McKeeman et al. (2002) have shown that the dosage of ethanol (1.8 mL/kg/d) similar to that used in the NTP chronic bio-

assay was sufficient to reduce hepatic betaine levels. Dosing with DEA had no further effect on hepatic betaine levels. Given the substantial interplay between choline and 1-carbon metabolism, it is highly likely that the hepatic effects of DEA observed in the NTP bioassay were exacerbated by the co-administration of ethanol. Furthermore, since the animals in the NTP chronic bioassay had free access to the unoccluded dermal application site during normal grooming activities, dosing would actually be a combination of dermal and oral exposure. Stott et al. (2000b) have shown that the blood DEA concentration in mice dosed dermally with access to the application site was 32% higher than in mice without access, suggesting a considerable portion of the dose was absorbed via the oral route. Since the liver receives the entire oral dose of DEA directly via the portal system, it may be at a greater risk than when DEA is administered dermally.

As discussed, two other modes of action have been suggested as possible alternatives to the intracellular choline deficiency hypothesis. One suggestion involves the in situ formation of the nitrosamine, NDELA. However, this is highly unlikely as studies found no NDELA in the plasma or urine of mice dosed with carcinogenic dosages of DEA in combination with an excess of nitrite to favor nitrosamine formation (Stott et al., 2000b). Another hypothesis involves the formation of DEA-containing phospholipids. It has been argued that aberrant phospholipids could alter signal transduction cascades, leading to aberrant cell signaling and cell cycling (NTP, 1999a). While DEA has been shown to incorporate into phospholipids (Artom et al., 1958; Mathews et al., 1995) and may be responsible for some of the pathogenicity of DEA, in particular anemia in rats, the weight of evidence suggests that it is not the principal mode of DEA-induced liver tumorigenesis in mice. Increased hepatocellular S-phase DNA synthesis has been linked to choline deficiency, either from DEA treatment or choline-deficient media or diets in cultured rat and mouse hepatocytes (Kamendulis and Klaunig, 2005) and liver of mice (Mellert et al., 2004), but not in human hepatocytes (Kamendulis and Klaunig, 2005), despite the metabolic incorporation of DEA into phospholipids in all three species (Mathews et al., 1995). Increased S-phase DNA synthesis is a well-recognized tumor risk factor (Goodman et al., 1991). Another risk factor, decreased gap junctional intercellular communication (Yamasaki, 1990), has also been observed in cultured rodent but not human hepatocytes treated with DEA or provided choline-deficient media (Kamendulis et al., 2004). Again, this was despite the fact that all these species are capable of incorporating DEA into phospholipids. Finally, no tumors were observed in rats chronically administered a dose level of DEA that was dermally irritating and capable of causing systemic effects (Melnick et al., 1994b), but not high enough to depress hepatic choline levels (Lehman-McKeeman et al., 2001). In contrast to the hypothesis of aberrant phospholipids, choline deficiency

is a well-characterized mode of action linking the cellular events of increases in S-phase DNA synthesis, disruption of DNA methylation, and decreased GJIC with tumorigenesis in rodents (Zeisel and Blusztajn, 1994; Abanobi et al., 1982). All these are characteristics of DEA-induced choline deficiency at tumorigenic dose levels in the NTP mouse bioassay (NTP, 1999a).

Since choline is an essential nutrient in all mammals, the proposed mechanism of DEA-induced choline deficiency is qualitatively applicable to humans. However, there are marked species differences in susceptibility to choline deficiency, with rats and mice being far more susceptible than other species including humans (Zeisel and Blusztajn, 1994). These differences are attributed to quantitative differences in the enzyme kinetics controlling choline metabolism. Rats and mice rapidly metabolize choline to betaine in the liver and it is likely that choline oxidase activity determines choline requirements and controls species sensitivity to choline deficiency (Sidransky and Farber, 1960). For example, choline oxidase activity is much lower in primates than rodents and primates are less sensitive to choline deficiency (Hoffbauer and Zaki, 1965). Humans have the lowest choline oxidase activity of all species and are generally refractory to choline deficiency, with evidence of choline deficiency observed only after prolonged fasting, significantly depressed liver function or deficient parenteral feeding (Zeisel and Blusztajn, 1994). It is noteworthy that there was no evidence of GJIC inhibition in human hepatocytes treated with DEA or cultured in choline-deficient media (Kamendulis et al., 2004).

The observation that DEA was carcinogenic in mice but not in rats (NTP, 1999a) has important implications for the overall evaluation of human cancer risk. Disposition data indicate that DEA is less readily absorbed across rat skin than mouse skin (Sun et al., 1996), and the resulting blood and tissue concentrations of DEA are at least 3 times lower in rats than in mice at similar dosages (Mathews et al., 1997). Lehman-McKeeman et al. (2002) has determined a NOEL for DEA-induced choline deficiency in mice (based on phosphocholine concentrations) to be 10 mg/kg/d, indicating that there is a critical concentration of DEA that must be attained in order to affect choline homeostasis. The lack of a carcinogenic response in rats suggests that it was unlikely that exposure to DEA reached this threshold level.

While most of the experimental evidence presented herein focuses on the effects of DEA in the liver, it is conceivable that a similar mode of action involving choline deficiency is responsible for the renal tubular adenomas observed in the NTP bioassay (NTP, 1999a). Choline deficiency is known to compromise renal functions and cause kidney toxicity in laboratory animals (Baxter, 1947; Michael et al., 1975; Zeisel and Blusztajn, 1994).

In conclusion, the experimental evidence is consistent with a mode of action involving the development of intra-

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## References

Abanobi  
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Barak  
choline  
Barber  
hepatic  
mice  
Barber  
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Pfeiffer  
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cellular choline deficiency, possibly exacerbated by co-administration of ethanol as the dosing vehicle and enhanced dose availability to the liver resulting from potential oral exposure. Since rodents are far more sensitive to choline deficiency than humans, it appears that the hepatocarcinogenic effect of DEA in mice is not predictive of similar susceptibility in humans.

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*Contact Person:* Raul A. Saavedra, PhD, Scientific Review Administrator, Scientific Review Branch, Division of Extramural Research, NINDS/NIH/DHHS, Neuroscience Center, 6001 Executive Blvd., Suite 3208, MSC 9529, Bethesda, MD 20892-9529. 301-496-9223.

*Name of Committee:* National Institute of Neurological Disorders and Stroke Initial Review Group, Neurological Sciences and Disorders A.

*Date:* February 16-17, 2003.

*Time:* 8:30 a.m. to 5 p.m.

*Agenda:* To review and evaluate grant applications.

*Place:* Holiday Inn Sunspree Resort, 7601 East Indian Bend Road, Scottsdale, AZ 85250.

*Contact Person:* Richard D. Crosland, PhD, Scientific Review Administrator, Scientific Review Branch, Division of Extramural Research, NINDS/NIH/DHHS, Neuroscience Center, 6001 Executive Blvd., Suite 3208, MSC 9529, Bethesda, MD 20892-9529. 301-496-9223.

*Name of Committee:* National Institute of Neurological Disorders and Stroke Initial Review Group, Neurological Sciences and Disorders K.

*Date:* February 16-17, 2003.

*Time:* 8:30 a.m. to 5 p.m.

*Agenda:* To review and evaluate grant applications.

*Place:* Holiday Inn Sunspree Resort, 7601 East Indian Bend Road, Scottsdale, AZ 85250.

*Contact Person:* Katherine M. Woodbury, PhD, Scientific Review Administrator, Scientific Review Branch, NINDS/NIH/DHHS, Neuroscience Center, 6001 Executive Blvd., Suite 3208, MSC 9529, Bethesda, MD 20892-9529. 301-496-9223.

*Name of Committee:* National Institute of Neurological Disorders and Stroke Initial Review Group, Neurological Sciences and Disorders B.

*Date:* February 20-21, 2003.

*Time:* 8:30 a.m. to 5 p.m.

*Agenda:* To review and evaluate grant applications.

*Place:* Washington Monarch Hotel, 2401 M Street NW., Washington, DC 2037.

*Contact Person:* W. Ernest Lyons, PhD, Scientific Review Administrator, Scientific Review Branch, NINDS/NIH/DHHS, Neuroscience Center, 6001 Executive Blvd., Suite 3208, MSC 9529 Bethesda, MD 20892-9529. 301-496-4056.

*Name of Committee:* National Institute of Neurological Disorders and Stroke Initial Review Group, Neurological Sciences and Disorders C.

*Date:* February 20-21, 2003.

*Time:* 8:30 a.m. to 5 p.m.

*Agenda:* To review and evaluate grant applications.

*Place:* Monarch Hotel, 2401 M Street, NW., Washington, DC 20037.

*Contact Person:* Andrea Sawczuk, DDS, PhD, Scientific Review Administrator, Scientific Review Branch, NINDS/NIH/DHHS, Neuroscience Center, 6001 Executive Blvd., Suite 3208, MSC 9529, Bethesda, MD 20892-9529. 301-496-0660. [sawczuka@ninds.nih.gov](mailto:sawczuka@ninds.nih.gov).

(Catalogue of Federal Domestic Assistance Program Nos. 93.853, Clinical Research Related to Neurological Disorders; 93.854, Biological Basis Research in the Neurosciences, National Institutes of Health, HHS)

Dated: January 14, 2003.

**LaVerne Y. Stringfield,**

*Director, Office of Federal Advisory Committee Policy.*

[FR Doc. 03-1366 Filed 1-21-03; 8:45 am]

**BILLING CODE 4140-01-M**

## DEPARTMENT OF HEALTH AND HUMAN SERVICES

### Food and Drug Administration

#### Establishment of Medical Device User Fee Rates for Fiscal Year 2003 and Interim Procedures; Correction

**AGENCY:** Food and Drug Administration, HHS.

**ACTION:** Notice; correction.

**SUMMARY:** The Food and Drug Administration (FDA) is correcting a correction notice that appeared in the **Federal Register** of January 10, 2003 (68 FR 1469). The document corrected a notice that appeared in the **Federal Register** of November 21, 2002 (67 FR 70228), which announced the rates and interim procedures for medical device user fees for fiscal year (FY) 2003. The November 21, 2002, document was inadvertently published with confusing language regarding the fee that must be paid by a small business that submits a 510(k) premarket notification for FDA review during FY 2003. The document intended to state that all 510(k)s submitted for FDA review during FY 2003 are subject to a standard fee of \$2,187, and that all submitters who are subject to a fee, including a small business, are required to pay this fee. This document corrects the error in the correction notice.

**ADDRESSES:** Persons with access to the Internet may obtain further information on the Medical Device User Fee and Modernization Act of 2002 at <http://www.fda.gov/cdrh/mdufma> or <http://www.fda.gov/cber/mdufma/mdufma.htm>.

**FOR FURTHER INFORMATION CONTACT:** Frank Claunts, Office of Management and Systems (HFA-20), Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857, 301-827-4427.

**SUPPLEMENTARY INFORMATION:** In FR Doc. 03-494, appearing in the **Federal Register** of January 10, 2003, the following correction is made:

1. On page 1469, in the second column, at the bottom of the page, item

3 is revised to read "On page 70229, in table 1, in the fourth column, in the last row, correct 'None in FY 2003' to read '2.187'."

Dated: January 16, 2003.

**Margaret M. Dotzel,**

*Assistant Commissioner for Policy.*

[FR Doc. 03-1381 Filed 1-16-03; 3:21 pm]

**BILLING CODE 4160-01-S**

## DEPARTMENT OF HEALTH AND HUMAN SERVICES

### Public Health Service

#### National Toxicology Program; Call for Public Comments on 10 Nominations, Proposed for Listing in the Report on Carcinogens, Eleventh Edition

##### Background

The National Toxicology Program (NTP) solicits final public comments on the nominations reviewed in 2002 for listing in the Report on Carcinogens, Eleventh Edition ("the Report"). This Report (previously known as the Annual Report on Carcinogens) is a Congressionally mandated listing of known human carcinogens and reasonably anticipated human carcinogens and its preparation is delegated to the National Toxicology Program by the Secretary, Department of Health and Human Services (DHHS). Section 301 (b) (4) of the Public Health Service Act, as amended, provides that the Secretary, (DHHS), shall publish a biennial report which contains a list of all substances (1) which either are known to be human carcinogens or may reasonably be anticipated to be human carcinogens; and (2) to which a significant number of persons residing in the United States (US) are exposed. The law also states that the reports should provide available information on the nature of exposures, the estimated number of persons exposed and the extent to which the implementation of Federal regulations decreases the risk to public health from exposure to these chemicals.

In 2002, ten nominations were reviewed for listing in the Eleventh Report. This review included two Federal and one non-government, scientific peer reviews and public comment and review. The three scientific review committees evaluated all available data relevant to the criteria for inclusion of candidate nominations in the Report. The criteria used in the review process and a detailed description of the review procedures, including the steps in the current formal review process, can be obtained from

the NTP home page Web site at <http://ntp-server.niehs.nih.gov/> or by contacting: Dr. C. W. Jameson, National Toxicology Program, Report on Carcinogens, MD EC-14, P.O. Box 12233, Research Triangle Park, NC 27709; phone: (919) 541-4096, fax: (919) 541-0144, e-mail: [jameson@niehs.nih.gov](mailto:jameson@niehs.nih.gov).

#### Public Comment Requested

The nominations reviewed in 2002 are provided in the following table with their Chemical Abstracts Services (CAS) Registry numbers (where available) and the recommendations from the three scientific peer reviews. The NTP will be making a final recommendation for these ten nominations for listing in, or

changing the current listing from *reasonably anticipated to be a human carcinogen* to the *known to be a human carcinogen* category in the Eleventh Report.

Background documents provided to the review committees and the public are available on the Internet in PDF-format at the address above. Hard copies of these documents are also available upon request from Dr. Jameson (contact information above). The NTP will review the recommendations from each of the review committees and consider the public comments received throughout the process in making decisions regarding the NTP recommendations to the Secretary, DHHS, for listing of the nominated

substances in the Report on Carcinogens, Eleventh Edition. The NTP solicits final public comment to supplement any previously submitted comments or to provide comments for the first time on any substance in the following table. Comments will be accepted for 60 days from the publication date of this announcement and should be directed to Dr. C. W. Jameson at the address provided above. Individuals submitting public comments are asked to include relevant contact information [name, affiliation (if any), address, telephone, fax, e-mail, and sponsoring organization (if any)].

Dated: January 10, 2003.

**Kenneth Olden,**

*Director, National Toxicology Program.*

#### SUMMARY OF RG1,<sup>1</sup> RG2<sup>2</sup> AND NTP BOARD SUBCOMMITTEE<sup>3</sup> RECOMMENDATIONS FOR THE NOMINATIONS REVIEWED IN 2002 FOR LISTING IN THE REPORT ON CARCINOGENS,<sup>4</sup> 11TH EDITION

Nomination/CAS No.	Primary uses or exposures	RGI action	RG2 action	NTP board subcommittee action
1-Amino-2,4-dibromoanthraquinone/ (81-49-2).	An anthraquinone-derived vat dye that is used in the textile industry.	Motion to list 1-amino-2,4-dibromoanthraquinone as reasonably anticipated to be a human carcinogen passed by unanimous vote (8/0).	Motion to list 1-amino-2,4-dibromoanthraquinone as reasonably anticipated to be a human carcinogen passed by unanimous vote (8/0).	Motion to list 1-amino-2,4-dibromoanthraquinone as reasonably anticipated to be a human carcinogen passed by unanimous vote (9/0)
Selected Heterocyclic Amines (three nominations): (1) MeIQ (2-Amino-3,4-dimethylimidazo- [4,5-f]quinoxaline)/ * (77094-11-2) (2) MeIQx (2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline)/ (77500-04-0) (3) PhIP (2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine)/(105650-23-5)	MeIQ, MeIQx, and PhIP are heterocyclic amines that are formed during heating or cooking and are found in cooked meat and fish.	Motion to list MeIQ as reasonably anticipated to be a human carcinogen passed by unanimous vote (6/0).  Motion to list MeIQx as reasonably anticipated to be a human carcinogen passed by a vote of 5 yes to 1 no. Negative vote cast because member felt data meet criteria to list as known human carcinogen.  Motion to list PhIP as reasonably anticipated to be a human carcinogen passed by a vote of 5 yes to 1 no. Negative vote cast because member felt data meet criteria to list as known human carcinogen.	Motion to list MeIQ as reasonably anticipated to be a human carcinogen passed by unanimous vote (8/0).  Motion to list MeIQx as reasonably anticipated to be a human carcinogen passed by unanimous vote (8/0).  Motion to list PhIP as reasonably anticipated to be a human carcinogen passed by unanimous vote (8/0).	Motion to list MeIQ as reasonably anticipated to be a human carcinogen passed by a vote of 8 yes, 0 no and 1 abstention. Abstention because member felt insufficient data for human exposure to list in the RoC.  Motion to list MeIQx as reasonably anticipated to be a human carcinogen passed by a unanimous vote (9/0).  Motion to list PhIP as reasonably anticipated to be a human carcinogen passed by unanimous vote (9/0).

SUMMARY OF RG1,<sup>1</sup> RG2<sup>2</sup> AND NTP BOARD SUBCOMMITTEE<sup>3</sup> RECOMMENDATIONS FOR THE NOMINATIONS REVIEWED IN 2002 FOR LISTING IN THE REPORT ON CARCINOGENS,<sup>4</sup> 11TH EDITION—Continued

Nomination/CAS No.	Primary uses or exposures	RG1 action	RG2 action	NTP board subcommittee action
Cobalt Sulfate/(10026–2401).	Cobalt sulfate is used in electroplating and electrochemical industries. It is also used as a coloring agent for ceramics, a drying agent in inks, paints, varnishes and linoleum, and has been added to animal feed as a mineral supplement.	Motion to list cobalt sulfate as reasonably anticipated to be a human carcinogen passed by unanimous vote (9/0).	Motion to list cobalt sulfate as reasonably anticipated to be a human carcinogen passed by a vote of 8 yes and 1 no. Negative vote cast because member felt exposure data in background document needed to be more specific for cobalt sulfate.	Motion to list cobalt sulfate as reasonably anticipated to be a human carcinogen passed by a vote of 8 yes to 1 no. Negative vote cast because member felt human exposure data not specific for cobalt sulfate.
Diethanolamine (DEA)/(111–42–2).	DEA is used in the preparation of surfactants used in liquid laundry, dishwashing detergents, cosmetics, shampoos, and hair conditioners; as a surface-active agent and corrosion inhibitor in metalworking fluids and as a dispersant in agricultural chemical formulations.	Motion <i>not</i> to list DEA in the RoC passed by a vote of 7 yes to 2 no. Negative votes cast because members felt data sufficient to list as reasonably anticipated to be a human carcinogen.	Motion <i>not</i> to list DEA in the RoC passed by unanimous vote (9/0).	Motion <i>not</i> to list DEA in the RoC passed by a vote of 8 yes to 1 no. Negative vote cast because member felt data sufficient to list as reasonably anticipated to be a human carcinogen.
Naphthalene (91–20–3) .....	Naphthalene is used as an intermediate in the synthesis of many industrial chemicals, and has been used as an ingredient in some moth repellants and toilet bowl deodorants, and to control lice on livestock and poultry.	Motion to list naphthalene as reasonably anticipated to be a human carcinogen passed by a vote of 6 yes to 1 no. Negative vote cast because member felt data not sufficient to list in the RoC.	The RG2 could not make a majority recommendation for either listing or not listing naphthalene in the RoC.	Motion to list naphthalene as reasonably anticipated to be a human carcinogen passed by unanimous vote (9/0).
Nitrobenzene (98–95–3) .....	Nitrobenzene is used mainly in the production of aniline, itself a major chemical intermediate in the production of dyes.	Motion to list nitrobenzene as reasonably anticipated to be a human carcinogen passed by unanimous vote (7/0).	Motion to list nitrobenzene as reasonably anticipated to be a human carcinogen passed by unanimous vote (7/0).	Motion to list nitrobenzene as reasonably anticipated to be a human carcinogen passed by unanimous vote (9/0).
Nitromethane (75–52–5) ...	Nitromethane is used in specialized fuels, in explosives and in the synthesis of nitromethane derivatives, pharmaceuticals, agricultural soil fumigants and industrial antimicrobials.	Motion to list nitromethane as reasonably anticipated to be a human carcinogen passed by unanimous vote (8/0).	Motion to list nitromethane as reasonably anticipated to be a human carcinogen passed by unanimous vote (9/0).	Motion to list nitromethane as reasonably anticipated to be a human carcinogen passed by unanimous vote (9/0).
4,4'-Thiodianiline (139–65–1).	4,4'-Thiodianiline has been produced commercially since the early 1940's as an intermediate of several diazo dyes.	Motion to list 4,4'-thiodianiline as reasonably anticipated to be a human carcinogen passed by a vote of 6 yes to 2 no. Negative votes cast because members felt there was not sufficient exposure to list in the RoC.	Motion to list 4,4'-thiodianiline as reasonably anticipated to be a human carcinogen passed by a vote of 6 yes to 3 no. Negative votes cast because members felt there was not sufficient exposure to list in the RoC.	Motion to list 4,4'-thiodianiline as reasonably anticipated to be a human carcinogen passed by a vote of 5 yes to 2 no with 2 abstentions. Negative votes and abstentions cast because members felt there was not sufficient exposure to list in the RoC.

<sup>1</sup> The NIEHS Review Committee for the Report on Carcinogens (RG1).<sup>2</sup> The NTP Executive Committee \* Interagency Working Group for the Report on Carcinogens (RG2).

\* Agencies from NTP Executive Committee represented on RG2 include: Agency for Toxic Substances and Disease Registry (ATSDR), Consumer Product Safety Commission (CPSC), Environmental Protection Agency (EPA), National Center for Environmental Health of the Centers for Disease Control and Prevention (NCEH/CDC), National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA), National Institute for Occupational Safety and Health/CDC (NIOSH/CDC), Occupational Safety and Health Administration (OSHA), National Cancer Institute of the National Institutes of Health (NCI/NIH), and National Institute of Environmental Health Sciences/NIH (NIEHS/NIH).

<sup>3</sup> The NTP Board of Scientific Counselors Report on Carcinogens Subcommittee (the External Peer Review Group).

<sup>4</sup> RoC—Report on Carcinogens.

[FR Doc. 03-1368 Filed 1-21-03; 8:45 am]  
BILLING CODE 4140-01-P

**DEPARTMENT OF HOUSING AND URBAN DEVELOPMENT**

[Docket No.FR-4815-N-01]

**Notice of Submission of Proposed Information Collection to OMB: Public Housing Agency—Lease Requirements, Recordkeeping Requirements**

**AGENCY:** Office of the Chief Information Officer, HUD.

**ACTION:** Notice.

**SUMMARY:** The proposed information collection requirement described below has been submitted to the Office of Management and Budget (OMB) for review, as required by the Paperwork Reduction Act. The Department is soliciting public comments on the subject proposal.

**DATES:** *Comments Due Date:* February 21, 2003.

**ADDRESSES:** Interested persons are invited to submit comments regarding this proposal. Comments should refer to the proposal by name and/or OMB approval number (2577-0006) and should be sent to: Lauren Wittenberg,

OMB Desk Officer, Office of Management and Budget, Room 10235, New Executive Office Building, Washington, DC 20503; Fax number (202)395-6974; e-mail *Lauren\_Wittenberg@omb.eop.gov*.

**FOR FURTHER INFORMATION CONTACT:**

Wayne Eddins, Reports Management Officer, AYO, Department of Housing and Urban Development, 451 Seventh Street, Southwest, Washington, DC 20410; e-mail *Wayne\_Eddins@HUD.gov*; telephone (202) 708-2374. This is not a toll-free number. Copies of the proposed forms and other available documents submitted to OMB may be obtained from Mr. Eddins.

**SUPPLEMENTARY INFORMATION:** The Department has submitted the proposal for the collection of information, as described below, to OMB for review, as required by the Paperwork Reduction Act (44 U.S.C. chapter 35). The notice lists the following information: (1) The title of the information collection proposal; (2) the office of the agency to collect the information; (3) the OMB approval number, if applicable; (4) the description of the need for the information and its proposed use; (5) the agency form number, if applicable; (6) what members of the public will be affected by the proposal; (7) how

frequently information submissions will be required; (8) an estimate of the total number of hours needed to prepare the information submission including number of respondents, frequency of response, and hours of response; (9) whether the proposal is new, an extension, reinstatement, or revision of an information collection requirement; and (10) the name and telephone number of an agency official familiar with the proposal and of the OMB Desk Officer for the Department.

This notice also lists the following information:

*Title of Proposal:* Public Housing Agency—Lease Requirements, Recordkeeping Requirements.

*OMB Approval Number:* 2577-0006.

*Form Numbers:* None.

*Description of the Need for the Information and Its Proposed Use:* Public Housing Agencies (PHA) are required to keep records for implementation of Federal regulations governing dwelling leases in public housing. The information is retained by the PHAs that manage public housing and is used for operating purposes.

*Respondents:* Individuals or households, State, local or tribal government.

*Frequency of Submission:* Annually.

	Number of respondents	Annual responses	×	Hours per response	=	Burden hours
Reporting Burden .....	3,330	3,330		48		158,400

*Total Estimated Burden Hours:* 158,400.

*Status:* Reinstatement, without change.

**Authority:** Section 3507 of the Paperwork Reduction Act of 1995, 44 U.S.C. 35, as amended.

Dated: January 14, 2003.

**Wayne Eddins,**

*Departmental Reports Management Officer, Office of the Chief Information Officer.*

[FR Doc. 03-1274 Filed 1-21-03; 8:45 am]

BILLING CODE 4210-72-P

**DEPARTMENT OF THE INTERIOR**

**Fish and Wildlife Service**

**Notice of Decision and Availability of Decision Documents on the Issuance of Permits for Incidental Take of Threatened and Endangered Species**

**AGENCY:** Fish and Wildlife Service, Interior.

**ACTION:** Notice of decision.

**SUMMARY:** Between November 14, 2001, and November 22, 2002, Region 1 of the Fish and Wildlife Service (we, the Service) approved 11 Habitat Conservation Plans (HCPs) and associated permits for the incidental take of threatened and endangered species, pursuant to section 10(a)(1)(B)

of the Endangered Species Act of 1973, as amended (Act). We also amended two HCPs and associated permits. In addition, we issued two permits for Safe Harbor Agreements and one permit for a Candidate Conservation Agreement with Assurances, pursuant to section 10(a)(1)(A) of the Act.

Copies of the permits and associated decision documents are available upon request. Charges for copying, shipping and handling may apply.

**ADDRESSES:** Documents are available from the Fish and Wildlife Service, 911 N.E. 11th Avenue, Portland, Oregon 97232.

**FOR FURTHER INFORMATION CONTACT:** If you would like copies of any of the above documents, please contact Shelly

**CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY  
OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT**

**SAFE DRINKING WATER AND TOXIC ENFORCEMENT ACT OF 1986  
(PROPOSITION 65)**

**NOTICE TO INTERESTED PARTIES**

**March 7, 2003**

**Decision Not to Proceed With the Listing of Diethanolamine  
Via the Authoritative Bodies Listing Mechanism**

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65 or the Act) requires the Governor to publish, and update at least annually, a list of chemicals known to the State to cause cancer or reproductive toxicity. The Act provides for administratively listing chemicals as known to the State to cause cancer or reproductive toxicity (Health and Safety Code Section 25249.8(b)) when a body considered to be authoritative by the state's qualified experts has formally identified it as causing cancer or reproductive toxicity. The National Toxicology Program, in addition to other bodies, has been identified as an authoritative body for purposes of the Act. The criteria for listing chemicals through the "authoritative bodies" mechanism are set forth in Title 22, California Code of Regulations (22 CCR) Section 12306.

As the lead agency for the implementation of Proposition 65, the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency examines documents released by authoritative bodies to identify chemicals for possible listing under Proposition 65. OEHHA identified documents produced by the National Toxicology Program (NTP, 1997a and b) supporting the possible listing of diethanolamine via the authoritative bodies mechanism. On February 5, 1999, OEHHA issued a "Request for Relevant Information" in the *California Regulatory Notice Register* (Register 99, No. 6) concerning the possible listing of diethanolamine as "known to cause cancer." As part of its commitment to public participation and external scientific peer review in its implementation of Proposition 65, OEHHA solicited, via that notice, information relevant to the evaluation of diethanolamine in the context of the Proposition 65 administrative listing regulatory criteria (22 CCR Section 12306). The notice announced the beginning of the public comment period for receiving written comments and a public forum (held March 2, 1999) for interested parties to present oral comments and to discuss the scientific data and other information relevant to a determination as to whether diethanolamine and other chemicals identified in the notice met the criteria for listing set forth in 22 CCR Section 12306.

OEHHA heard and received in writing substantive public comment on the possible listing of diethanolamine. Considerable scientific information has been released subsequent to the release of the National Toxicology Program report on diethanolamine (NTP, 1997a). This new information has been considered by OEHHA in light of 22 CCR Section 12306(f). Because it is not clear that the scientific criteria for listing under the authoritative bodies

mechanism have been met, OEHHA has decided not to proceed with the administrative listing of diethanolamine under Proposition 65.

**References:**

National Toxicology Program (NTP, 1997a). *Toxicology and Carcinogenesis Studies of Diethanolamine (CAS No. 111-42-2) in F344/N Rats and B6C3F<sub>1</sub> Mice (Dermal Studies)*. Board Draft. NTP Technical Report Series No. 478 NTIS Publication No. 97-3968. US Department of Health and Human Services, NTP, Research Triangle Park, NC.

National Toxicology Program (NTP, 1997b). *Summary Minutes from Peer Review of Draft Technical Reports of Long-Term Toxicology and Carcinogenesis Studies by the Technical Reports Review Subcommittee on December 9-10, 1997*. NTP, Research Triangle Park, NC.

# Diethanolamine and Phenobarbital Produce an Altered Pattern of Methylation in GC-Rich Regions of DNA in B6C3F1 Mouse Hepatocytes Similar to That Resulting from Choline Deficiency

Ammie N. Bachman,\* Lisa M. Kamendulis,† and Jay I. Goodman\*<sup>1</sup>

\*Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824; and

†Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Received November 22, 2005; accepted December 28, 2005

DNA methylation is an epigenetic mechanism regulating transcription, which when disrupted, can alter gene expression and contribute to carcinogenesis. Diethanolamine (DEA), a non-genotoxic alkanolamine, produces liver tumors in mice. Studies suggest DEA inhibits choline uptake and causes biochemical changes consistent with choline deficiency (CD). Rodents fed methyl-deficient diets exhibit altered methylation of hepatic DNA and an increase in liver tumors, e.g., CD causes liver tumors in B6C3F1 mice. We hypothesize that DEA-induced CD leads to altered methylation patterns which facilitates tumorigenesis. B6C3F1 hepatocytes in primary culture were grown in the presence of either 4.5 mM DEA, 3 mM Phenobarbital (PB), or CD media for 48 h. These concentrations induced comparable increases in DNA synthesis. PB, a nongenotoxic rodent liver carcinogen known to alter methylation in mouse liver, was included as a positive control. Global, average, DNA methylation status was not affected. The methylation status of GC-rich regions of DNA, which are often associated with promoter regions, were assessed via methylation-sensitive restriction digestion and arbitrarily primed PCR with capillary electrophoretic separation and detection of PCR products. DEA, PB, and CD treatments resulted in 54, 63, and 54 regions of altered methylation (RAMs), respectively, and the majority were hypomethylations. A high proportion of RAMs (72%) were identical when DEA was compared to CD. Similarly, 70% were identical between PB and CD. Altered patterns of methylation in GC-rich regions induced by DEA and PB resemble that of CD and indicate that altered DNA methylation is an epigenetic mechanism involved in the facilitation of mouse liver tumorigenesis.

**Key Words:** DNA methylation; diethanolamine; choline deficiency; phenobarbital; GC-rich regions.

Diethanolamine (DEA), an alkanolamine, is used in industrial applications such as textile processing, industrial gas purification, and preparation of agricultural chemicals. In addition, fatty acid condensates synthesized from DEA are

found in numerous consumer products such as cosmetics, soaps, and detergents (Knaak *et al.*, 1997). Widespread human exposure to DEA prompted the National Toxicology Program (NTP) to examine its carcinogenic potential. Dermal applications of DEA in 95% ethanol for two years led to significant increases in the incidence and multiplicity of liver tumors in male and female B6C3F1 mice, but not F344 rats. Recently, DEA-induced increases in liver cell proliferation were observed *in vitro*. Importantly, this effect was specific to F344 rats and B6C3F1 mice and not observed with human hepatocytes (Kamendulis and Klaunig, 2005). Based on *in vitro* genetic toxicity studies DEA and/or its metabolites are not mutagenic (NTP, 1999), suggesting that it induces a tumorigenic response via a secondary, non-genotoxic mechanism(s).

Similar in structure to ethanolamine and choline, two essential precursors for the synthesis of phospholipids, DEA is incorporated into hepatic phospholipids, perhaps disrupting regulation of choline and 1-carbon metabolism. Furthermore, DEA can inhibit the uptake of choline leading to intracellular deficiency, even if there is an adequate amount of choline in the diet (Lehman-McKeeman and Gamsky, 1999). Deficiencies in the major dietary sources of methyl groups, specifically, choline and methionine, lead to hepatocarcinogenesis in rodents (Henning and Swendseid, 1996; Poirier, 1994). Choline deficiency (CD) causes hepatocyte proliferation and apoptosis (Albright *et al.*, 1996; Ziesel, 1996). In particular, CD in rodents, including B6C3F1 mice, in the absence of known carcinogens, increases liver tumor development (Newberne *et al.*, 1982, Newberne and Rodgers, 1986).

Diets lacking in choline and methionine result in altered levels of S-adenosyl methionine (SAM), and S-adenosyl homocysteine (SAH). SAM is the main methyl donor for a variety of methylation reactions including DNA methylation (Ziesel, 1996). In effect, methyl deficiency decreases SAM and increases SAH shifting the proportionality towards SAH which is a feedback inhibitor of DNA methyltransferases and, therefore, the SAM/SAH ratio is a determinant of the extent of methylation (Shivapurkar and Poirier, 1983). In B6C3F1 mice, dermal application of DEA resulted in decreased levels of

<sup>1</sup> To whom correspondence should be addressed at Michigan State University, B-440 Life Sciences Bldg., East Lansing, MI 48824. Fax: (517) 353-8915. E-mail: goodman3@msu.edu.

SAM, increased levels of SAH, and a reduction in phosphocholine, the intracellular storage form of choline, which are all consistent with previous reports of biochemical changes associated with CD which leads to methyl deficiency (Lehman-McKeeman *et al.*, 2002). Indeed, deficiencies in methionine and choline have been shown to lead to global, average hypomethylation of DNA in the livers of B6C3F1 mice (Counts *et al.*, 1996).

It has been hypothesized that alteration of the epigenome, specifically DNA methylation, is a mechanism underlying DEA-induced tumorigenesis in B6C3F1 mouse liver (Kamendulis and Klaunig, 2005; Lehman-McKeeman and Gamsky, 1999). Methylation of cytosines to produce 5-methyl cytosine is a well characterized, heritable, epigenetic mark (Feinberg, 2001). The majority of 5-methyl cytosine occurs at cytosines 5' to guanine. These CpG dinucleotides are not evenly distributed throughout the genome (Bird, 2002), but are concentrated in GC-rich promoter regions of genes and transposable elements typically being located within CpG islands which are stretches of DNA, at least 200 bp in length that possess a 50% or greater GC content and a higher proportion of CpG dinucleotides than expected (Gardiner-Garden and Frommer, 1987). Decreases in methylation are associated with increases in gene transcription while increases in methylation are associated with decreases in gene transcription (Jones and Laird, 1999).

Phenobarbital (PB) is a non-genotoxic promoter of rodent liver tumors (Whysner *et al.*, 1996). Increased cell proliferation and altered DNA methylation are likely involved in tumor promotion (Goodman and Watson, 2002). Following PB administration increases in DNA synthesis occur in B6C3F1 liver, indicating enhanced cell proliferation, as early as 1–2 weeks (Klaunig, 1993). Additionally, PB induces more global hypomethylation in the liver tumor-prone B6C3F1 mouse, as compared to the relatively resistant C57BL/6, mouse (Counts *et al.*, 1996). A more critical look at this has shown that PB induces hypermethylation in selected GC-rich regions of DNA in addition to global hypomethylation demonstrating a non-random disruption of the epigenome (Watson and Goodman, 2002).

Using B6C3F1 mouse hepatocytes in primary culture, we have examined GC-rich regions of the genome for changes in methylation in response to treatment with DEA, choline deficient media, or PB. The hypothesis being tested is that DEA-induced CD leads to altered methylation patterns which facilitate mouse liver tumorigenesis. The effects of DEA and PB on DNA methylation status was ascertained and compared with changes produced by CD. Specifically, we have assessed global (average) methylation and evaluated the methylation status of GC-rich regions of the genome using an arbitrarily primed PCR approach.

## MATERIALS AND METHODS

**Mouse hepatocytes.** Male B6C3F1 mice, 6–8 weeks old, obtained from Harlan Sprague-Dawley were housed in a facility at Indiana University School

TABLE 1  
Summary of Replicative DNA Synthesis

Treatment	Labeling index <sup>a,b</sup>
Control	1.84 ± 0.09
Diethanolamine	7.51 ± 0.21 <sup>c</sup>
Choline deficiency	7.14 ± 0.17 <sup>c</sup>
Phenobarbital	7.61 ± 0.24 <sup>c</sup>

<sup>a</sup>Labeling Index: percentage of BrdU positive nuclei in a minimum of 1000 hepatocytes.

<sup>b</sup>Labeling index is expressed as mean ( $n = 3$ ) percent ± SE.

<sup>c</sup>Statistically different from control. Statistical significance was determined via ANOVA, Tukey's  $p < 0.05$ .

of Medicine (IUSM) and cared for in accordance with the University's animal use and care guidelines. Hepatocytes were isolated by a two-step *in situ* collagenase perfusion (Klaunig *et al.*, 1981), cultured, and treated with 4.5 mM DEA, 0.0898 mg/l choline, or 3 mM PB for 48 h at IUSM. Isolated hepatocytes from each of three animals per dosing group were divided and cultured in two plates. DNA was obtained from 8–10 × 10<sup>6</sup> cells using TRIzol Reagent, following the manufacturer's guidelines.

**DNA synthesis.** Replicative DNA synthesis was measured according to the method of James and Roberts (1996). BrdU (20 mM final concentration) was added to cell cultures during the last 16 h of culture. Cells, 1 × 10<sup>6</sup> hepatocytes/60 mm culture dish, were washed and fixed with methanol. Incorporated BrdU was localized using an anti-BrdU antibody followed by a peroxidase linked secondary antibody and a DAB substrate. Replicative DNA synthesis was measured by scoring the percentage of BrdU positive nuclei in a minimum of 1000 hepatocytes. Statistical significance was determined via a Randomized Complete Block Design ANOVA, post-hoc test, Tukey's,  $p < 0.05$ .

**SssI global (average) methylation assay.** This assay allows for methylation at the 5' position of cytosine at every unmethylated CpG site in DNA via the enzyme *SssI* methylase using [Methyl-<sup>3</sup>H] S-adenosyl methionine (SAM) as the methyl donor, as described previously (Counts *et al.*, 1996). Global DNA methylation can be determined by the amount of <sup>3</sup>H-methyl groups incorporated into DNA, since there is an inverse relationship between incorporation of radioactivity and the degree of methylation. Each DNA sample was

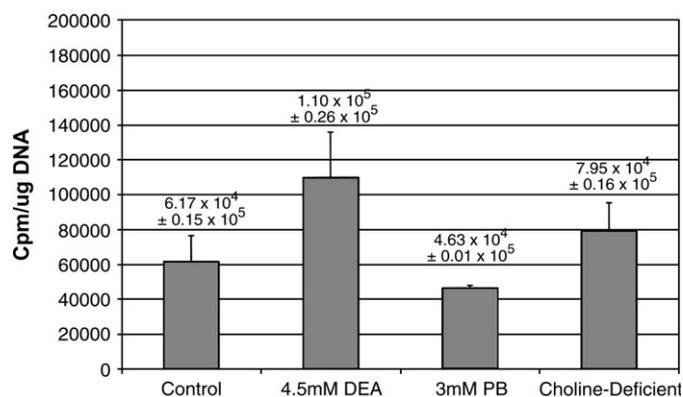
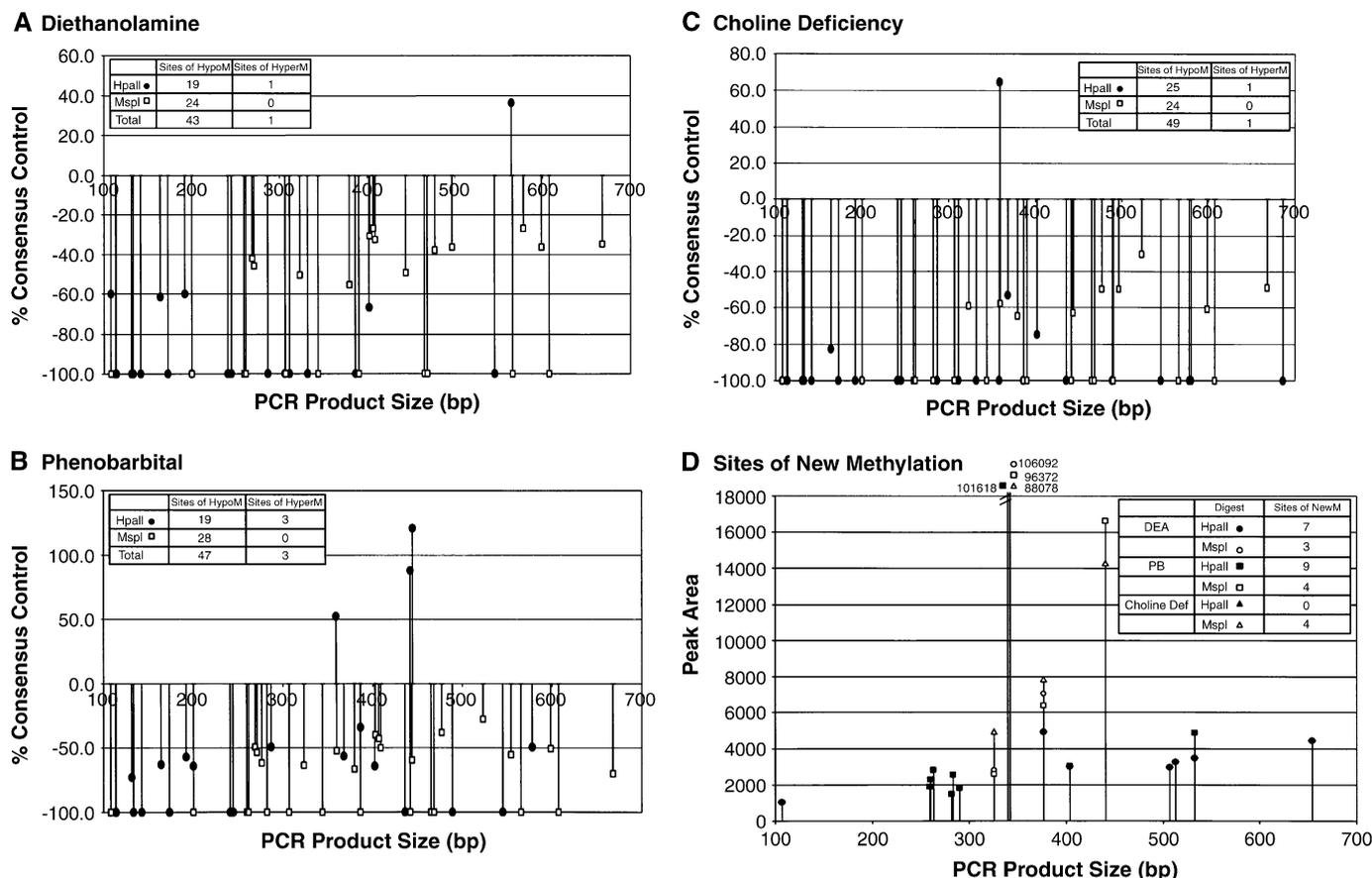


FIG. 1. Global methylation status in DNA from primary mouse hepatocytes. Global methylation of DNA isolated from primary mouse hepatocytes treated for 48 h with 4.5 mM DEA, choline deficient media or 3 mM phenobarbital is presented. Each bar represents the mean CPM/ug DNA of three animals ± SE. DEA, choline deficiency, and phenobarbital treatment were statistically ( $p < 0.05$ ) no different from control.



**FIG. 2.** GC-rich DNA methylation status in primary B6C3F1 mouse hepatocytes. *RsaI/HpaII* (closed symbols) and *RsaI/MspI* (open symbols) digestion and subsequent AP-PCR was performed on DNA isolated from B6C3F1 mouse hepatocytes treated with either DEA (A), phenobarbital (B), or choline deficient media (C) for 48 h. Regions of hypomethylation were prevalent across all treatments. (D) Regions of new methylation resulting from treatment are shown in terms of the peak area for each PCR product size. Four regions of new methylation whose peak areas exceeded the scale of the chart were labeled above the chart with their corresponding peak area values. Tables tallying the regions of altered methylation for each treatment are shown as an inset in each chart. Regions of hypo-, hyper-, and new methylation determined by the data are expressed in terms of the treated mean for each PCR product size as a percent of the control mean for each PCR product size. All changes projecting below the x-axis represent decreases in methylation (hypomethylation) while all those above the x-axis represent increases in methylation (hypermethylation). All 100% hypomethylations are considered to be significant, and only the hypermethylations and partial hypomethylations that were statistically significantly different from control values (Student's *t*-test,  $p < 0.05$ ) are depicted.

incubated with 0.75  $\mu$ g of DNA per 5 replicates with 2.25 units *SssI* Methylase, 1.5  $\mu$ Ci [ $^3$ H-methyl] SAM and reaction buffer (10 mM Tris, 120 mM NaCl, 10 mM EDTA, 1 mM DTT, pH 7.9) to volume. Reactions were spotted onto DE81 ion exchange filters and washed with 25 ml 0.5M phosphate buffer, 2 ml 70% ethanol and 2 ml 100% ethanol and allowed to dry before scintillation counting. All results are expressed as cpm/ $\mu$ g DNA.

#### Arbitrarily Primed PCR and Capillary Electrophoresis

A comparison of data obtained from DNA isolated from control and treated tissue permits the simultaneous detection of treatment-related increased methylation (hypermethylation, more methylation in a region that was methylated in control), decreased methylation (hypomethylation, less methylation in a region that was methylated in control), and new methylations (methylation in regions that were not methylated in control). Therefore, the procedure we have developed provides an in depth picture of treatment related altered methylation is provided.

**Restriction digests.** DNA samples are subjected to double digests with restriction enzymes: (1) a methylation insensitive enzyme, and (2) a methylation sensitive enzyme. *RsaI* is the methylation insensitive enzyme which is used

initially to cut DNA into fragments in order to facilitate complete digestion by the second enzyme, a methylation-sensitive restriction enzyme. The methylation sensitive enzymes used in this study were *MspI* and *HpaII*. Both recognize 5'CCGG 3' sites, and cut between the cytosine and guanine. *MspI* will not restrict DNA if the external cytosine is methylated, while *HpaII* will not restrict DNA if the internal cytosine is methylated. Both *RsaI/MspI* and *RsaI/HpaII* double digests were employed.

**Arbitrarily primed PCR (AP-PCR) and capillary electrophoresis.** PCR is performed on restriction digests using a single arbitrary primer 5' AACCT-CACCCTAACCCCGG 3' (Gonzalzo *et al.*, 1997), that was modified by having it fluorescently labeled at the 5' end with HEX (purchased from Integrated DNA Technologies). This primer was designed to bind well to GC-rich regions and the 5'CCGG 3' sequence at its 3' end increases the probability of primer annealing to the *HpaII* and *MspI* restriction site. This allows for detection of methylation at the site of primer annealing and between sites of primer annealing. Each PCR product is viewed as representing a GC-rich region of the genome. PCR products were purified, using a sephadex G50 superfine matrix, and separated via capillary electrophoresis, using an ABI 3700 Genetic Analyzer (Genomics Technology Support Facility [GTSF] at Michigan State

TABLE 2  
Summary of GC-Rich Regions of Altered Methylation (RAMs)

Treatment	Digest	RAMs Hypomethylation <sup>a</sup>	RAMs Hypermethylation <sup>b</sup>	RAMs "New" methylation <sup>c</sup>	Total
Diethanolamine	HpaII	19	1	7	
	MspI	24	0	3	
Total <sup>d</sup>		43	1	10	54
Phenobarbital	HpaII	19	3	9	
	MspI	28	0	4	
Total <sup>d</sup>		47	3	13	63
Choline deficiency	HpaII	25	1	0	
	MspI	24	0	4	
Total <sup>d</sup>		49	1	4	54

<sup>a</sup>Hypomethylated RAMs include both statistically significant ( $p < 0.05$ ) decreases and 100% decreases.

<sup>b</sup>Hypermethylated RAMs are only those increases which are statistically significant ( $p < 0.05$ ).

<sup>c</sup>New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.

<sup>d</sup>Total RAMs including hypomethylations, hypermethylation, and new methylations for the combined digests are reported for each treatment.

University). Base pair markers are run simultaneously with the samples in order to accurately size the PCR products. The results represented as size of PCR products, in base pairs, and their corresponding peak areas are analyzed using the Excel program. A consensus, average, peak area for each PCR product reporting in control and treated groups is prepared, and the consensus control and treated peak areas at a specific PCR product are compared. This permits us to detect treatment-related: (1) hypomethylations which include both 100% decreases and decreases which are statistically significant when compared to control, (2) hypermethylations which are increases which are statistically significant when compared to control, and (3) new methylations which are indicated by the formation of a PCR product following treatment which was not formed under control conditions. Significance is determined via a Student's  $t$ -test,  $p < 0.05$ . Analysis of the data includes the following assumptions: (1) each separate PCR product of a defined size represents a distinct region of the genome, (2) a region can include one or more recognition sequences for the specific methylation-sensitive restriction enzyme employed located between the annealing sites of the up- and down-stream primers; thus, the amount of each PCR product formed can be viewed as representing an "average" of the methylation status of the particular recognition sequences located between the up- and down-stream primers, and (3) changes in the amount of each PCR product represents the altered methylation status of a particular GC-rich region of DNA. A detailed account of the AP-PCR, capillary electrophoresis method, including the data analysis steps are provided as Supplementary Data.

## RESULTS

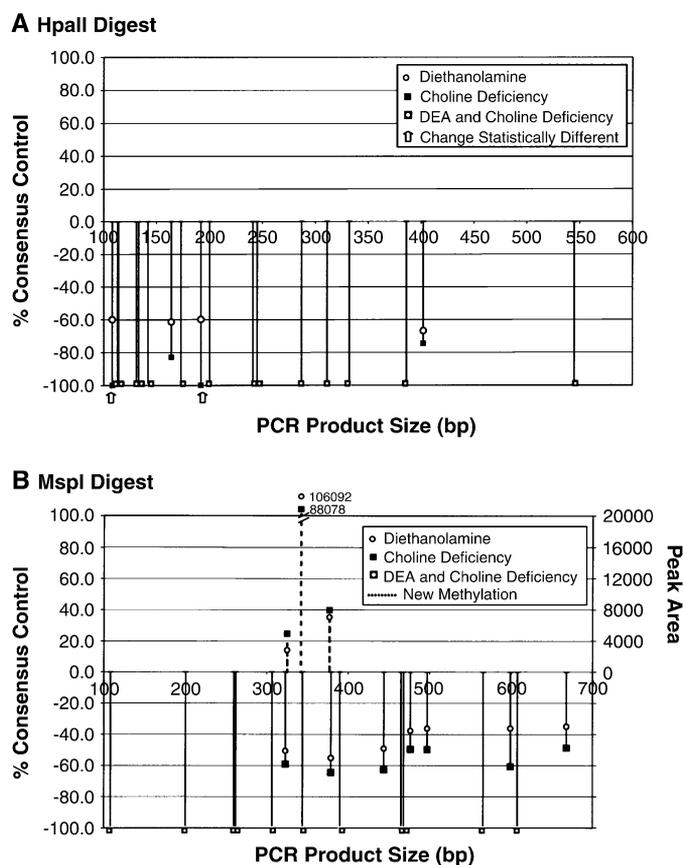
In order to provide an equivalent baseline from which we could compare the effects of DEA, PB, or CD on the methylation status of DNA in B6C3F1 hepatocytes, we selected concentrations (4.5 mM, 3 mM, and 0.098 mg/l for DEA, PB, and CD media, respectively) that produced equivalent increases in DNA synthesis during the 48 h culture period (Table 1). DEA or PB treatment as well as culture in CD media did not affect global, average methylation status (Fig. 1).

Analysis of GC-rich regions of DNA provided a more detailed picture of altered methylation patterns than simply evaluating global, average methylation. DEA treatment re-

sulted in 43 regions of hypomethylation, which composed 80% of the total aberrant regions detected within GC-rich areas of DNA (Fig. 2A). Of these, 26 (60%) exhibited a 100% decrease (i.e., a complete loss of methylation) at those regions. The large degree of significant decreases in methylation (both partial and complete hypomethylation) was approximately equal in number at both the external and internal cytosine of 5'-CCGG-3' regions based upon the results of the RsaI/MspI and RsaI/HpaII digests. In comparison, relatively few regions of methylation increased with only 1 hypermethylation and 10 new regions of methylation (Figs. 2A and 2D). Here increases were mainly detected via the RsaI/HpaII digest indicating a preference for altered methylation of the internal cytosine within the recognition sequence.

PB produced a pattern of altered methylation similar to DEA. The largest proportion of altered regions, 75%, were hypomethylations (Fig. 2B) with 49% of the total decreases exhibiting a complete loss of methylation. Increases in methylation included 3 regions of hypermethylation and 13 regions of new methylation (Figs. 2B and 2C). Similar to the results obtained from DEA treatment, there was a bias towards increased methylation at the internal cytosine within the 5'CCGG 3' recognition sequence.

DNA isolated from hepatocytes maintained in CD media exhibited the greatest number of regions where 5' Me-C content was either partially or completely decreased (Fig. 2C). Methylation was lost completely in 37 of the 49 (76%) total hypomethylated regions. Very few increases in methylation were observed; 1 site of hypermethylation was identified via the RsaI/HpaII digest and 4 regions of new methylation were identified via the RsaI/MspI digest (Figs. 2C and 2D) indicating that of the small number of increases, most occurred at the external cytosine in contrast to increases induced by DEA or PB which occurred mainly at the internal cytosine. The predominate



**FIG. 3.** Comparison of diethanolamine and choline deficiency induced aberrant GC-rich methylation patterns. Regions of altered methylation induced by DEA and choline deficiency are compared for the RsaI/HpaII (A) and RsaI/MspI (B) digestion. PCR products of identical size formed in both the control and treatment groups were considered to be common regions of altered methylation. These common regions of hypo-, hyper-, and new methylations are represented. For the majority of common regions of aberrant methylation, the magnitude and direction of change induced by DEA and choline deficiency were statistically no different (one-way ANOVA,  $p < 0.05$ ). At only two common regions identified by the RsaI/HpaII digest, were the magnitudes of change statistically different. In each case, choline deficiency induced a greater loss of methylation than DEA. All changes projecting below the x-axis represent decreases in methylation (hypomethylation) while all those above the x-axis represent increases in methylation (hypermethylation). All 100% hypomethylations are considered to be significant, and only the hypermethylations and partial hypomethylations that were statistically significantly different from control values (Student's *t*-test,  $p < 0.05$ ) are depicted.

alteration in methylation patterns was a decrease in methylation at multiple regions within GC-rich regions. PB produced the greatest degree of altered methylation with 63 total altered regions. DEA and CD treatment were strikingly similar with 54 total altered regions (Table 2).

Due to the overall similarity in patterns of altered methylation among the different treatments, a more refined approach to analyzing and comparing the data was employed. Changes occurring at identical PCR product sizes between two treatments were considered common regions of altered methylation.

Figure 3 depicts the 39 regions of altered methylation in common between DEA and CD treatments. The magnitudes of change at only 2 regions of the 39 total regions were statistically different (Fig. 3 and Table 3). Of the 44 common regions of altered methylation between PB and CD treatments, only 5 regions differed statistically in magnitude (Fig. 4 and Table 3). The patterns of altered methylation produced by DEA and PB were 72 and 70% similar, respectively, to that of CD demonstrating the high degree of similarity (Table 3). Unique changes elicited by DEA and PB were few in numbers (Table 4).

## DISCUSSION

We have developed and applied a novel procedure for analyzing altered methylation in GC-rich regions of the genome, including CpG islands. Simple in design, this technique employs methylation sensitive restriction digestion of DNA, arbitrarily primed PCR amplification, and electrophoretic separation of PCR products to provide a detailed, quantitative overview of the extent of treatment-related disruption of methylation throughout the genome. Comparably, the strength and utility of our technique lies in its ability to simultaneously identify increases, decreases, and new methylations within multiple, distinct regions of the genome. This provides a sensitive, quantitative method which reproducibly detects the extent of treatment-related altered patterns of methylation.

There are a variety of techniques for analyzing changes in methylation within a particular gene. Methylation specific PCR, including variations such as MethyLight and HM MethyLight can be effectively employed for these applications (Cottrell and Laird, 2003). Other procedures include combined bisulfite restriction analysis (COBRA) which assesses the methylation status of particular CpG sites (Xiong and Laird, 1997) and the enzymatic regional methylation assay for determining changes in methylation between two primers designed for a targeted region (Galm *et al.*, 2002). These are excellent methods for evaluating specific genes. However, their utility is limited when one wants to discern the extent to which a particular treatment might disrupt normal methylation patterns, e.g., in this situation a gene-by-gene approach would be too cumbersome.

The methylation-sensitive amplified fragment length polymorphism (AFLP) technique allows for comparative genome wide scanning of methylation status via fingerprinting techniques and has recently been adapted to a DNA microarray hybridization technique (Yamamoto and Yamamoto, 2004). This procedure requires a custom microarray panel and a complex approach to data analysis. Global, average methylation analysis via SssI methyltransferase (Balaghi and Wagner, 1993) is straightforward, but limited in scope; increases in methylation in one portion of the genome may balance out decreases in other areas. The combined AP-PCR capillary electrophoresis technique described in this article affords the

TABLE 3  
Common Regions of Altered Methylation (RAMs): Comparison of Diethanolamine (DEA) or Phenobarbital (PB) with Choline Deficient (Choline Def) Treatment

	Common RAMs with decreased methylation <sup>a</sup>	Common RAMs with increased methylation <sup>b</sup>	Total RAMs in common	Percent similarity
DEA vs. Choline Def				
Complete hypomethylation <sup>c</sup>	25	—	39/54 <sup>i</sup>	72%
Partial hypomethylation <sup>d</sup>	9	—		
New methylation <sup>e</sup>	—	3		
Magnitude of change statistically different <sup>g,h</sup>	2	—		
PB vs. Choline Def				
Complete hypomethylation <sup>c</sup>	23	—	44/63 <sup>i</sup>	70%
Partial hypomethylation <sup>d</sup>	12	—		
New methylation <sup>e</sup>	—	3		
Hypermethylation <sup>f</sup>	—	1		
Magnitude of change statistically different <sup>g,h</sup>	5	—		

Note. Data are summarized from Figures 2 and 3.

<sup>a</sup>Total RAMs exhibiting decreased methylation (i.e., complete hypomethylations and partial hypomethylations) that are in common between DEA and Choline Def or PB and Choline Def treatments.

<sup>b</sup>Total RAMs exhibiting increased methylation (i.e., hypermethylations and new methylations) that are in common between DEA and Choline Def or PB and Choline Def.

<sup>c</sup>Complete hypomethylation indicates a complete or 100% loss of methylation.

<sup>d</sup>Partial hypomethylations are statistically significant ( $p < 0.05$ ) decreases as compared to control.

<sup>e</sup>New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.

<sup>f</sup>Hypermethylations are statistically significant ( $p < 0.05$ ) increases as compared to control.

<sup>g</sup>Significance was based on a one-way ANOVA,  $p < 0.05$ .

<sup>h</sup>Choline Def showed a significantly greater extent of hypomethylation than DEA and PB treatment at the number of RAM indicated.

<sup>i</sup>Total RAMs are reported from Table 2.

ability to assess altered DNA methylation (increases, decreases, and new methylations) in multiple GC-rich regions of the genome simultaneously and quantitatively. Furthermore, it is highly appropriate under situations when the research question being asked is, "Does a particular treatment cause disruption of normal patterns of DNA methylation and to what extent does this occur?" With this methodology we have assessed DEA, CD, and PB induced alteration of methylation in B6C3F1 mouse hepatocytes.

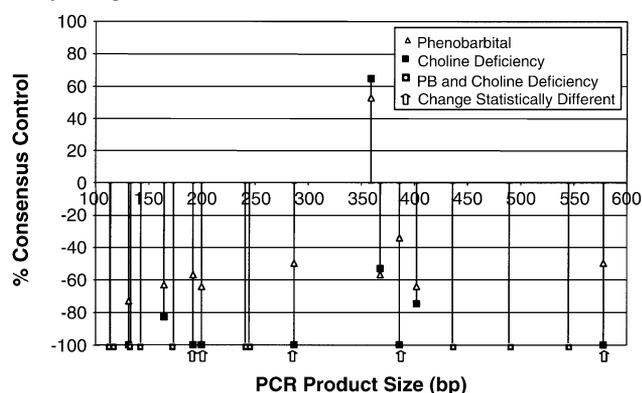
The ability of DEA to alter methylation *in vitro* in B6C3F1 mouse hepatocytes was investigated as a proposed non-genotoxic mode of action of the compound's ability to cause carcinogenesis in mouse liver. Since high rates of DNA synthesis might compromise the capacity to maintain normal methylation patterns leading to mis-regulated gene expression patterns doses were selected based on induction of comparable increases in cell proliferation. Therefore, we were able to directly compare and analyze changes in methylation employing a common baseline.

Several factors work in concert to sustain normal methylation levels. These include the maintenance (Dnmt 1) and *de novo* DNA methyltransferases (Dnmt3a and 3b), demethylases and the availability of both SAM and methyl groups. For example, Dnmt1 is the maintenance methyltransferase responsible for

methylating newly synthesized daughter strands of DNA; this ensures the heritability of the methylation pattern (Hermann *et al.*, 2004). Altered patterns of methylation, specifically hypomethylation, may arise when the activity of Dnmt1 does not increase with enhanced rates of DNA synthesis. Alternatively, the same effect could be observed if SAM does not provide a sufficient supply of methyl groups, (i.e., methyl deficiency depletes the availability of methyl groups), to maintain the up regulated Dnmt1 activity. DNA methylation patterns are also under the influence of demethylases (e.g., MBD2) which can decrease the level of 5-methyl cytosine when cells are not synthesizing DNA (Detich *et al.*, 2003). Thus, indicating that DNA methylation is reversible. Importantly, SAM directly inhibits MBD2 and, therefore, diminished formation of SAM during a state of methyl deficiency could relieve the inhibition of demethylase activity and facilitate hypomethylation of DNA (Detich *et al.*, 2003). As hypothesized, DEA, by inducing cellular choline depletion, contributes to perturbation of 1-carbon metabolism, leading to decreased availability of methyl groups, impaired formation of SAM, and disruption of normal DNA methylation patterns.

Assessment of global (average) methylation status and methylation of GC-rich regions of DNA were performed. Global, average levels of methylation following treatment with

## A HpaII Digest



## B MspI Digest

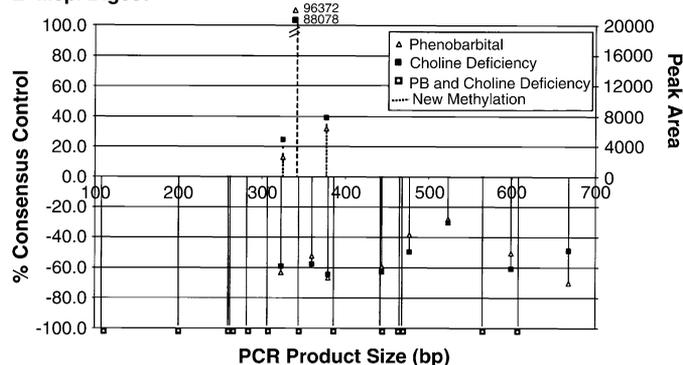


FIG. 4. Comparison of phenobarbital and choline deficiency induced aberrant GC-rich methylation patterns. Regions of altered methylation induced by PB and choline deficiency are compared for the RsaI/HpaII (A) and RsaI/MspI (B) digestion. PCR products of identical size formed in both the control and treatment groups were considered to be common regions of altered methylation. These common regions of hypo-, hyper-, and new methylations are represented. For most regions, the magnitude and direction of change induced by PB and choline deficiency were statistically no different (one-way ANOVA,  $p < 0.05$ ). The magnitudes of decrease for five common regions, identified by the RsaI/HpaII digest, were statistically different. In each case, choline deficiency induced a greater loss of methylation than PB. All changes projecting below the x-axis represent decreases in methylation (hypomethylation) while all those above the x-axis represent increases in methylation (hypermethylation). All 100% hypomethylations are considered to be significant, and only the hypermethylations and partial hypomethylations that were statistically significantly different from control values (Student's  $t$ -test,  $p < 0.05$ ) are depicted.

DEA, CD media, and PB were comparable to control (Fig. 1). In a previous study global hypomethylation of hepatic DNA was observed following treatment of B6C3F1 mice with PB *in vivo* (Counts *et al.*, 1996). These data are not necessarily incompatible with the current observation because total liver DNA was examined following treatment for 1 week, or more, as compared to a 48 h *in vitro* exposure of isolated hepatocytes. The current data could indicate that (1) global methylation levels are unaffected by DEA, CD, and PB or (2) approximately equal levels of methylation increases and decreases are occurring simultaneously in multiple regions of the genome.

TABLE 4  
Unique Regions of Altered Methylation (RAMs):  
Diethanolamine (DEA) or Phenobarbital (PB) as Compared  
to Choline Deficient Treatment

	DEA	PB
Complete hypomethylation <sup>a</sup>	1	1
Partial hypomethylation <sup>b</sup>	6	7
Hypermethylation <sup>c</sup>	1	2
New methylation <sup>d</sup>	7	9
Total RAM <i>not</i> in common	15/54 <sup>e</sup>	19/63 <sup>e</sup>
Percent difference	28%	30%

Note. Unique RAMs denotes all RAM which were not in common between DEA and choline deficiency or PB and choline deficiency.

<sup>a</sup>Complete hypomethylation indicates a complete or 100% loss of methylation.

<sup>b</sup>Partial hypomethylations are statistically significant ( $p < 0.05$ ) decreases as compared to control.

<sup>c</sup>Hypermethylations are statistically significant ( $p < 0.05$ ) increases as compared to control.

<sup>d</sup>New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.

<sup>e</sup>Total RAMs are reported from Table 2.

In light of the fact that the SssI procedure for evaluating global methylation assesses the methylation status of all cytosines 5' to guanines (whether or not they are located in GC-rich regions of DNA), the second possibility underlies the importance of specifically examining GC-rich regions for a more detailed picture of overall altered methylation.

Within GC-rich regions of DNA, hypomethylation was the predominant alteration induced by DEA, PB, and CD. Hypomethylation in the promoter regions of genes is associated with increased gene expression (Jones and Baylin, 2002). Critical losses of methylation in the promoter regions of oncogenes such as *c-jun* and *c-myc*, *CDNK3* (cyclin-dependent kinase inhibitor 3), and *c-Ha-ras*, have been demonstrated (Niculescu *et al.*, 2004; Tao *et al.*, 2000). Hypomethylation associated overexpression of *c-jun* and *c-myc* was observed in livers promoted with dichloroacetic and trichloroacetic acid, both of which are considered non-genotoxic carcinogens (Tao *et al.*, 2000). Human neuroblastoma cells cultured in CD media showed loss of methylation in the promoter region of the *CDNK3* gene, an important regulator of cell cycle progression, and up-regulation of expression. In addition, genetic instability via activation of transposable elements (Roman-Gomez *et al.*, 2005), elevated mutation rates (Chen *et al.*, 1998), and chromosomal instability (Eden *et al.*, 2003) have all been associated with hypomethylated DNA. Methyl deficiency in rats induced irreversible global DNA hypomethylation in rat liver which supported a role for loss of methylation during the cancer initiation and or promotion stages of hepatocarcinogenesis (Progribny *et al.*, 2005). These studies emphasize and support

our view that DNA hypomethylation is a mechanism involved in tumor promotion (Counts and Goodman, 1995) and the data presented in the current article support the hypothesis that DEA, CD and PB treatment act by this mechanism to produce mouse liver tumors.

Altered methylation status of cytosines within the CpG dinucleotide is most commonly investigated; however, methylation of CpNpG and non-CpG sites also exists. In particular, the role of altered methylation at CpCpG sites has not been thoroughly investigated. There are three possible states of methylation of the CpCpG sites analyzed. These include (1) <sup>m</sup>CpCpG, methylation of the external cytosine, (2) Cp<sup>m</sup>CpG, methylation of the internal cytosine, and (3) <sup>m</sup>Cp<sup>m</sup>CpG, methylation of both the internal and external cytosine. Our results show that loss of methylation status at both <sup>m</sup>CpG and <sup>m</sup>CpCpG sites occurs with approximately equal frequency suggesting that factors affecting the methylation status of <sup>m</sup>CpG sites also act on <sup>m</sup>CpCpG sites. Studies evaluating non-CpG methylation have mainly focused on CpA, CpT, and CpC methylation. However, one particular study proposed a biological role for methylation of both cytosines within CpCpG sites. Methylation of both cytosines within CpCpG sites has been reported to prevent binding of Sp1, an important transcription factor, to its target cis element thereby contributing to abnormal regulation of gene expression (Clark *et al.*, 1997; Inoue and Oishi, 2005). Effects due to methylation of only the external cytosine were not reported. This stresses the importance of a broad and critical analysis of both CpG and non-CpG methylation during the promotion stage of tumorigenesis.

We have demonstrated remarkable similarities between the DEA, CD, and PB treatment related disruption of methylation patterns in B6C3F1 mouse hepatocytes grown *in vitro* during a short 48 h exposure. This indicates that a common mechanism is shared by all three treatments. The extreme similarity between patterns of altered methylation in GC-rich regions due to DEA and CD supports the notion that DEA indirectly depletes the pool of methyl groups needed for methylation of cytosine by inhibiting choline uptake into cells (Lehman-McKeeman and Gamsky, 1999). The resulting hypomethylation mimics that of dietary CD. Dietary PB has been shown to cause global hypomethylation (Counts *et al.*, 1996), and hypermethylation, along with some decreased methylation, in GC-rich regions of DNA (Watson and Goodman, 2002) in the livers of B6C3F1 mice after 2 and 4 weeks of administration. Therefore, continued exposure to the promoting stimuli may lead to progressive changes in methylation including hypomethylations, hypermethylations (treatment-related increases in methylation in RAMs that were methylated in control), and new methylations (treatment-related methylation of RAMs that were not methylated in control), which accrue in a stepwise manner to contribute to tumorigenesis. This is consistent with the view that a variety of alterations in methylation contribute to carcinogenesis (Counts and Goodman, 1995), and that there are progressive alterations of methylation during the trans-

formation process (Watson *et al.*, 2003). Hence, altered methylation, initially hypomethylation, is a likely epigenetic, non-genotoxic mode of action underlying the abilities of DEA, PB, and CD to promote the development of mouse liver tumors.

## SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>. Supplementary Data contains a detailed description of the materials and methods for the arbitrarily primed PCR and capillary electrophoretic approach employed assessing methylation status in GC-rich regions throughout the genome. In addition, data organization and analysis, including statistical calculations performed using the Excel program, are explained in detail.

## ACKNOWLEDGMENTS

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## DEA Reference List for Prop 65 Evaluation of Cocamide Diethanolamine

Busch, Jon [Jon\_Busch@americanchemistry.com]

**Sent:** Thursday, January 20, 2011 8:15 AM  
**To:** Oshita, Cynthia@OEHHA  
**Cc:** Busch, Jon [Jon\_Busch@americanchemistry.com]  
**Attachments:** DEA Reference List for OEH-1.doc (40 KB)

Dear Ms. Oshita:

**RE: Request for Comments on Cocamide Diethanolamine (coconut oil acid diethanolamine condensate; CAS No. 68603-42-9).**

I am hereby submitting a reference list on diethanolamine (DEA, CAS # 111-42-2), which may have some use and relevance for the upcoming Prop 65 review of cocamide diethanolamine. The references on diethanolamine (DEA) in the attached list are focused primarily on DEA carcinogenicity and DEA mechanistic studies, with a few DEA pharmacokinetics studies also presented. I have electronic copies of all the references cited in this reference list, should OEHHA need a copy.

As you are likely aware, IARC reviews cocamide diethanolamine on February 15-22, 2011.

Kind Regards,

Jon

**Jonathon T. Busch**

**Manager, Alkanolmaines Panel**

**Director, Chemical Products & Technology Division**

**American Chemistry Council** | 700 – 2<sup>nd</sup> Street NE | Washington, DC | 20002

[jon\\_busch@americanchemistry.com](mailto:jon_busch@americanchemistry.com)

Office: (202) 249-6725 | Cell: (703) 439-7076

[www.americanchemistry.com](http://www.americanchemistry.com)

On [October 22, 2010](#), OEHHA published a notice in the California Regulatory Notice Register (Register 10 No. 43-Z) soliciting information which may be relevant to the evaluation of cocamide diethanolamine (coconut oil acid diethanolamine condensate) (CAS No. 68603-42-9) under consideration for possible listing within the context of the Proposition 65 administrative listing regulatory criteria in Title 27 of the California Code of Regulations section 25306.

The publication of the notice initiated a 60 day public comment period which would have closed on December 21, 2010. OEHHA has received a request from interested parties seeking an extension of the comment period to allow for the submission of complete and relevant scientific information for cocamide diethanolamine (coconut oil acid diethanolamine condensate). **OEHHA hereby extends the public comment period for this chemical until 5 p.m., Tuesday, February 1, 2011.**

We encourage you to submit comments in electronic form, rather than in paper form. Comments transmitted by e-mail should be addressed to [coshita@oehha.ca.gov](mailto:coshita@oehha.ca.gov). Comments submitted in paper form may be mailed, faxed, or delivered in person to the addresses below:

Ms. Cynthia Oshita

Office of Environmental Health Hazard Assessment

Mailing Address: P.O. Box 4010, MS-19B

Sacramento, California 95812-4010

Fax: (916) 323-8803

Street Address: 1001 I Street

Sacramento, California 95814

## **Reference List for Diethanolamine (DEA, CAS # 111-42-2)**

Reference List Prepared by:

Alkanolamines Panel of the American Chemistry Council

Contact: Jon Busch

Manager, Alkanolamines Panel

Director, Chemical Products & Technology Division

[jon\\_busch@americanchemistry.com](mailto:jon_busch@americanchemistry.com)

American Chemistry Council

700 – 2<sup>nd</sup> Street NE

Washington, DC 20002

USA

Office: (202) 249-6725 | Cell: (703) 439-7076

[www.americanchemistry.com](http://www.americanchemistry.com)

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