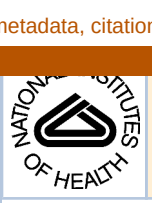

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Evaluation of the Potential of Triethanolamine to Alter Hepatic Choline Levels in Female B6C3F1 Mice

W. T. Stott[*,1], **B. J. Radtke**[*], **V. A. Linscombe**[*], **M-H Mar**[†], and **S. H. Zeisel**[†]

* *Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan 84674; and*

† *Department of Nutrition, School of Public Health and School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599–7641*

Abstract

Triethanolamine (TEA), a widely used nongenotoxic alcoholamine, has recently been reported to cause an increased incidence of liver tumors in female B6C3F1 mice, but not in males nor in Fischer 344 rats. Choline deficiency induces liver cancer in rodents, and TEA could compete with choline uptake into tissues. The potential of TEA to cause choline deficiency in the liver of these mice as a mode of tumorigenesis was investigated. Groups of female B6C3F1 mice were administered 0 (vehicle) or a maximum tolerated dosage (MTD) of 1000 mg/kg/day TEA (Trial I) and 0, 10, 100, 300, or 1000 mg/kg/day TEA (Trial II) in acetone vehicle via skin painting 5 days/week for 3 weeks. Female CDF® rats were also administered 0 or an MTD dosage of 250 mg/kg/day TEA (Trial II) in a similar manner. No clinical signs of toxicity were noted, and upon sacrifice, levels of hepatic choline, its primary storage form, phosphocholine (PCho), and its primary oxidation product, betaine, were determined. A statistically significant decrease in PCho and betaine, was observed at the high dosage (26–42%) relative to controls and a dose-related, albeit variable, decrease was noted in PCho levels. Choline levels were also decreased 13–35% at the high dose level in mice. No changes in levels of choline or metabolites were noted in treated rats. A subsequent evaluation of the potential of TEA to inhibit the uptake of ^{3}H-choline by cultured Chinese Hamster Ovary Cells revealed a dose-related effect upon uptake. It was concluded that TEA may cause liver tumors in mice via a choline-depletion mode of action and that this effect is likely caused by the inhibition of choline uptake by cells.

Keywords

triethanolamine; mechanism; choline deficiency

Triethanolamine (TEA; CAS 102-71-6) has found widespread usage in a number of industrial and consumer products (Knaak *et al*., 1997). In general, TEA is relatively nontoxic to animals following acute and subchronic administration and has not been found to be carcinogenic in a number of bioassays conducted in several species using oral or dermal routes of administration (reviewed by Knaak *et al*., 1997; NTP, 2003). Chronic ingestion of dosages calculated to be greater than 3000 mg/kg/day TEA by B6C3F1 mice has not caused increases in tumor formation (Konishi *et al*., 1992). TEA has also been uniformly negative in a number of *in vitro* and *in vivo* genotoxicity assays. Despite this, recently TEA has been shown to cause an increased incidence of liver adenomas in female B6C3F1 mice following chronic administration of 100, 300, and 1000 (maximum tolerated dosage; MTD) mg/kg/day via skin painting (NTP, 2003). No increased incidence in tumors occurred in male mice dosed with up

[1] To whom correspondence should be addressed. E-mail: wstott@dow.com..

to 2000 mg/kg/day TEA, nor were increased liver tumors observed in male or female Fischer 344 rats using a similar study design at dosages up to MTDs of 125 and 250 mg/kg/day, respectively (NTP, 1999a, 2003).

The secondary amine analogue of TEA, diethanolamine (DEA), is a minor impurity in most TEA formulations. DEA has also been reported to cause liver tumors in mice, albeit a more pronounced response, but not in rats under a similar study design (NTP, 1999b) as used in the TEA studies. Subsequent investigations of the possible mode of tumorigenesis of DEA resulted in the elucidation of a DEA-induced choline-deficiency-based mechanism for tumor formation in treated mice (Lehman-McKeeman *et al.*, 2002; Lehman-McKeeman and Gamsky, 2000; Stott *et al.*, 2000a). Significantly, the mechanism by which choline deficiency can cause tumors in rodents has been well characterized, and a general lack of sensitivity of higher mammals to develop this deficiency has been identified (Lehman-McKeeman *et al.*, 2002; Stott *et al.*, 2000a; reviewed by Zeisel and Blusztajn, 1994). DEA was shown both to upset a primary route of choline synthesis in cells by its displacement of ethanolamine in synthesis of phosphatidylethanolamine and to interfere with the uptake of choline across the cell membrane (Barbee and Hartung, 1979; Lehman-McKeeman and Gamsky, 1999). Species-specific increases in cell proliferation were characterized by Kamendulis *et al.* 2002, 2003, and tumorigenic dosages of DEA also depressed the methylation potential of liver in mice (Lehman-McKeeman *et al.*, 2002; Stott *et al.*, 2000b).

Treatment with a variety of alkylamines and alkanolamines has been reported to alter phospholipid synthesis and choline incorporation into phospholipids of cultured hepatocytes and/or cell lines (Akesson, 1977; Borman, 1982; Glaser *et al.*, 1974). These findings suggest the possibility that TEA, like DEA, may inhibit the uptake of choline by liver in treated mice, resulting in choline deficiency and a similar mode of tumorigenesis as with DEA. Thus, this study examined the potential of TEA to alter choline levels in mice and rats at dose levels and means of administration utilized in the NTP (1999a; 2003) bioassays.

MATERIALS AND METHODS

Chemicals—Two samples of TEA were used, one supplied by The Dow Chemical Company (Seadrift, TX; Lot QB1955R8S4) for use in the first mouse trial and the other a generous gift of the National Toxicology Program (RTI International, Research Triangle Park, NC; Lot 7G-60/02) for use in the second mouse and the rat trials. Both had greater than 99% purity; however, analyses identified differences in DEA impurity levels, 0.04% and 0.45%, respectively. Choline was obtained from Sigma Chemical Company (St. Louis, MO). Choline chloride [methyl-^{3}H] was obtained from New England Nuclear (Boston, MA). Dose solutions of TEA were prepared gravimetrically, and fresh solutions were utilized each week of the dosing periods.

Animals and dosing—Female B6C3F1/CrlBR® (B6C3F1) mice and CDF® (Fischer 344/CrlBR) (CDF) rats were obtained from Charles River Laboratories Inc. (Portage, MI). The laboratory is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and the Laboratory Animal Care and Use Committee approved all procedures used in the study. Animals were housed one per cage in appropriate stainless steel cages in rooms designed to maintain adequate conditions (temperature, humidity, and photocycle). Animals were provided LabDiet® Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, MO) in pelleted form in a hanging feeder and municipal water from a pressure-activated nipple-type watering system *ad libitum*. This diet contains approximately 1800 ppm choline. Following acclimation, animals (8–12 weeks of age) were stratified using preexposure body weights and were randomly assigned to treatment groups using a computer program and identified via subcutaneously implanted transponders

(BioMedic Data Systmens, Seaford, Delaware). Animals were observed twice daily to evaluate health status and weighed weekly during the study.

An area on the back of each mouse or rat, between the scapulae and stretching posterior approximately halfway to the ileum, was clipped free of hair at least 24 h prior to initiation of dosing. This area was reclipped over the course of the dosing period, as needed, at least 24 hours prior to resumption of dosing. Care was taken to avoid abrasian or nicking of skin. Dosing was as described by NTP (1999a; 2003), by applying ("painting") solutions or vehicle (acetone) directly on the skin using a blunt syringe. Dose solutions in acetone vehicle were applied at a volume of 4 ml/kg (250 mg/ml) in the first mouse trial, 2 ml/kg (5–500 mg/ml) in the second mouse trial, and 0.5 ml/kg (500 mg/ml) in the rat trial. The exposure site was not occluded, nor was any restraining device employed to prevent grooming. An evaluation of potential irritancy of the dosing solutions to the application site skin was made weekly using a standard evaluation scheme.

In vivo *study design and choline analysis*—Two *in vivo* evaluations of the effect of dermally administered TEA upon hepatic choline and metabolites were undertaken utilizing dosages used in the NTP bioassay (NTP, 2003). In the first trial, groups of ten mice were administered 0 (acetone vehicle) or 1000 mg/kg/day TEA 5 days/week for 3 weeks. In the second trial, groups of eight mice were administered 0 (acetone), 10, 100, 300, or 1000 mg/kg/day, and rats were administered 0 (acetone) or 250 mg/kg/day TEA 5 days/week for 3 weeks. Dose levels encompassed those used in the NTP bioassays of TEA in mice (NTP, 2003) and rats (NTP, 1999a). Animals were sacrificed on the last day of exposure approximately 2–4 hours following the last dermal dosing. Mice and rats were rapidly anesthetized with CO_2, decapitated, and briefly exsanguinated, and livers were rapidly excised and snap-frozen in liquid nitrogen. Livers were stored at −80°C and shipped on dry ice to Dr. Steven Zeisel's laboratory at the University of North Carolina (Chapel Hill, NC) for analysis.

Hepatic choline and its metabolites, PCho and betaine, were quantitated using the liquid chromatography (LC) electrospray ionization (ESI) isotope dilution mass spectrometry method outlined by Koc *et al*. 2002. Briefly, weighed samples of liver were extracted with a methanol/chloroform solution, appropriate deuterated internal standards were added, and the aqueous fraction components were separated using LC, followed by ESI and monitoring of selected ions by mass spectrometry. Data collection and analysis was as described using XCalibur® software.

***^{3}H-Choline uptake* in vitro—**The cell line CHO-K_1-BH_4, originally obtained from Dr. Abraham Hsie (Oak Ridge National Laboratory, Oak Ridge, TN) was used. Cells were grown in Ham's F-12 nutrient mix fortified with 5% (V/V) heat-inactivated, dialyzed fetal bovine serum, antibiotics and antimycotics (penicillin G, 100 units/ml; streptomycin sulfate, 0.1 mg/ml; fungizone, 0.25 μg/ml), and an additional 2 mM L-glutamine under a 5% CO_2 atmosphere at 37°C. CHO cells were seeded at a density of 2×10^5/well in 1 ml of culture medium in 24-well culture plates and cultured overnight. Dose solutions were prepared gravimetrically by mixing high purity TEA or DEA in culture medium, adjusting the pH to 7.4 to achieve concentrations of approximately 0.67–3.4 mM TEA and 0.05–1.9 mM DEA. The highest concentration of DEA as an impurity in TEA at the concentrations tested was 0.2 mg/ml. ^{3}H-Choline (5 mCi/well) was added directly to actively growing (nonconfluent) cultures, which were then incubated under a 5% CO_2 atmosphere at 37 °C for an additional 10 min. The final concentration of choline in the media of treated cultures was approximately 367 mM. Choline uptake was stopped by the addition of 1 ml of ice-cold PBS/well. Excess ^{3}H-choline was removed by repeated washing of the wells with PBS. Cells were then trypsinized, pelleted by centrifugation at 2000 × *g* for 5 min, and solubilized in 0.1 N sodium hydroxide. Wells containing media only served as controls. The protein concentration of each solubilized culture

was determined using a BCA assay (Pierce, Rockford, IL), and ^{3}H content was quantitated using liquid scintillation counting. Uptake was calculated as total recovered radioactivity per mg protein.

Statistics—Data were evaluated by Bartlett's test (alpha = 0.01) for equality of variances, followed by a parametric analysis of variance (ANOVA) (Steel and Torrie, 1960). If significant at alpha = 0.05, a Dunnett's test (alpha = 0.05) was conducted (Winer, 1971). In addition, a linear orthogonal polynomial contrast was used to test for linear trend in dose-response data using a significance value of 0.05 (Trial II mouse data) (Winer, 1971).

RESULTS

Clinical Observations and Body Weights

No effects of dosing upon the clinical appearance, body weights, or weight gains of mice or rats were noted (data not shown). In addition, no evidence of dermal irritation of dosing solutions was noted during the dosing period.

In Vivo *Choline and Choline-Related Metabolites*

In the initial mouse trial (Trial I), a 1000 mg/kg/day TEA dosage caused statistically identified decreases in betaine (26%) and PCho (35%) levels relative to vehicle treated controls (Fig. 1). A smaller decrease in hepatic choline concentration (13%) was also observed, which was not statistically identified. In a subsequent dose-response experiment (Trial II mice), all three measured parameters were statistically identified by Trend Test as changing over the dose range, despite a noticeable degree of variability in the data (Table 1). PCho levels were decreased by 18–20% at 100–300 mg/kg/day and by 42% at 1000 mg/kg/day compared to controls. Hepatic betaine levels were also decreased across most dosages, with minimal levels observed at the high dosage (29% decrease), and choline levels of high-dose-group mice were depressed by 35% compared to controls. Pairwise statistically significant changes were limited to high-dose groups. Administration of 250 mg/kg/day TEA to male CDF rats failed to cause a significant change in any measured parameter.

In Vitro *Choline Uptake*

TEA caused a statistically identified decrease in the uptake of ^{3}H-choline by growing CHO cells. A dose-related decrease in uptake occurred from 0.67 mM to 1.34 mM concentrations, reaching a maximal inhibition of approximately 60–70% of control at 1.34 to 3.4 mM over the 10-min dosing period (Fig. 2). A more pronounced response to DEA was observed, with a dose-related decrease in ^{3}H-choline uptake observed from 0.048 to 0.15 mM, reaching a maximal inhibition of approximately 75% of control at 0.19 to 1.9 mM.

DISCUSSION

In an attempt to elucidate the mode by which TEA may cause tumors in female mice, TEA in an acetone vehicle was repeatedly applied to the shaved backs of female mice, in a method similar to that used to dose animals in the NTP (2003) skin-painting bioassay. Systemic exposure to TEA administered via skin painting is assured because TEA is relatively well absorbed by mouse skin. Approximately 90% of a 1000 mg/kg dose of TEA applied to the backs of C3H/HeJ mice was reportedly absorbed within 24 h in the absence of any grooming activity (Stott *et al.*, 2000c). In addition, a portion of painted TEA is likely ingested during grooming activity in the absence of occlusion of the application site. Ingestion of a portion of painted DEA by mice was demonstrated by Stott *et al.* 2000a. Skin-painting administration is thus more accurately described as a combination of dermal absorption and oral ingestion. Interestingly, an 82-week oral bioassay of TEA conducted by Konishi *et al.* 1992 employing

B6C3F1 mice at much higher dosages (up to 3000 mg/kg/day) than those used in the NTP skin painting study did not result in liver tumor formation. While a number of study-specific variables may have accounted for this difference (animal suppliers, diets, duration of dosing), these results and those of other negative bioassays point out the relatively inconsistent and weak tumorigenic potential of this chemical in test animals.

Administration of TEA to B6C3F1 mice in the present study was clearly shown to decrease hepatic levels of the primary storage form of choline, PCho, and its oxidation product, betaine (Pelech and Vance, 1984). In both mouse Trial I and II, significant decreases in PCho levels (35 and 42%, respectively) and betaine levels (26 and 29%, respectively) occurred at 1000 mg/kg/day relative to controls. Decreases in choline levels also occurred at this high dosage, but to a much greater degree (35% decrease) in the second trial than in Trial I (13% decrease). A dose-related, albeit somewhat variable, decrease in PCho levels was also observed in the second trial, ranging from 18% to 42% over the three dose levels utilized in the NTP bioassay (100, 300, and 1000 mg/kg/day). The variability in measured parameters is not surprising, given the rapid metabolism of choline (Pelech and Vance, 1984) and the potential variability inherent in dosing via skin painting. However, it is noteworthy that control values of choline, PCho, and betaine were similar to those of more recent reports (Lehman-McKee-man *et al.*, 2002; Stott *et al.*, 2000b). Significantly, no consistent evidence of decreases in measured parameters occurred in mice administered 10 mg/kg/day TEA, nor were changes observed in female CDF rats administered a maximum tolerated and yet nontumorigenic dose level of 250 mg/kg/day.

Decreases in hepatic PCho, betaine, and choline levels observed at 1000 mg/kg/day in the second trial were more pronounced than in the first trial. Trial I involved administration of a relatively pure sample of TEA having only approximately 0.04% DEA impurity. The resulting dosage of DEA in Trial I was thus only approximately 0.4 mg/kg/day DEA, well below the no effect level of 10 mg/kg/day DEA for demonstrable changes in choline levels in mice under a similar study design by Lehman-McKeeman *et al.* 2002. In the subsequent Trial II, a sample of TEA containing approximately 0.45% DEA was used to provide comparative data. A generous gift from NTP, this latter TEA was used in the most recent NTP (2003) bioassay and resulted in coadministration of a maximal 4.5 mg/kg/day DEA dose. While still below the no effect level for DEA in the Lehman-McKeeman *et al.* 2002 study, an additive effect with that of TEA in the present study appears likely.

The loss of betaine in TEA-treated mice in the present study is particularly significant because it represents a loss of a choline metabolite that is central to the synthesis of *S*-adenosylmethionine (SAM), a principle methylating agent for bio-synthetic pathways and maintenance of critical gene methylation patterns. Loss of hepatic betaine and SAM has also been identified in choline-deficient rodents (Zeisel and Blusztajn, 1994). Similar changes in hepatic betaine levels were observed by Lehman-McKeeman *et al.* 2002. Data from this latter study revealed an association between an approximately 20% decrease in hepatic betaine levels and decreases in SAM levels in B6C3F1 but not C57BL/6 mice treated with ethanol and/or a tumorigenic dose level of DEA. The former strain is relatively sensitive, and the latter strain resistant to spontaneous liver tumor formation, suggesting a possible link between these factors in the relatively sensitivity B6C3F1 mouse strain.

While sharing a mode of tumorigenic action, it is obvious that overall TEA is less potent than DEA at causing choline depression in mice. Lehman-McKeeman *et al.* 2002 reported hepatic PCho levels depressed by 2–50% in B6C3F1 mice administered tumorigenic dose levels of 40–160 mg/kg/day DEA for 4 weeks. A depression of approximately 80% in hepatic PCho levels was obtained at 160 mg/kg/day DEA by Stott *et al.* (2000a,b) under similar conditions. Not surprisingly, the choline depressive responses obtained with these two compounds reflect their relative tumorigenic potencies. TEA resulted primarily in an elevation in single

occurrences of benign liver tumors in one sex of B6C3F1 mice, while DEA caused multiple occurrences of both benign and malignant tumors in both sexes of mice (NTP 1999b; 2003).

DEA is known to affect choline levels by inhibiting the uptake of choline by cells (Lehman-McKeeman and Gamsky, 1999) and by competition with ethanolamine in phosphatidylethanolamine synthesis (Barbee and Hartung, 1979; Lehman-McKeeman and Gamsky, 1999; Mathews *et al.*, 1995), effectively limiting a primary biosynthetic source of choline in mature animals. Effects of TEA upon the uptake of ^{3}H-choline by CHO-K1 cells were observed in the present study. Consistent with the relative potency in depleting cellular choline pools *in vivo*, TEA was much less potent than DEA at inhibiting ^{3}H-choline uptake, with maximal effects of approximately 60–70% of control obtained at 1.34–2.68 mM TEA versus approximately 74% of control at 0.19 mM DEA. Effects of TEA were not attributed to the small amounts of DEA impurity in the test material. The concentrations of DEA needed to cause a minimal effect upon ^{3}H-choline uptake in DEA assays was 36- to180-fold higher than the levels in TEA-treated cultures. In addition, TEA is unlikely to alter *de novo* synthesis of choline for several reasons. No conversion to DEA was observed in mice dosed with ^{14}C-TEA, and unlike DEA, no accumulation of TEA in liver tissues of mice occurred, as would be expected if metabolic incorporation at the expense of ethanolamine into phosphatidylethanolamine was occurring (Mathews *et al.*, 1997; D. Rick, unpublished data; Stott *et al.*, 2000c). Lehman-McKeeman (personal communication) has also demonstrated a lack of effect of TEA treatment upon synthesis rates of phosphatidylethanolamine in cultured CHO-K1 cells. Finally, the much broader spectrum of organ toxicity observed in test animals with DEA than with TEA suggests a fundamental difference in the metabolism of the two materials (reviewed by Knaak *et al.*, 1997; NTP, 2003).

It was concluded that TEA induces changes in liver of mice consistent with a choline-deficiency mode of tumorigenesis, as elucidated by S. Zeisel and coworkers (reviewed by Zeisel and Blusztajn, 1994) and most recently demonstrated for DEA in mice by Lehman-McKeeman *et al.* 2002 and Stott *et al.* 2000a. This effect appears to be a property of TEA exclusive of any DEA impurity; however the latter may also contribute to choline depletion in treated mice. Tumor formation requires the chronic depression of choline pools resulting in a relatively well-characterized sequence of biochemical, cytological, and genomic changes. Significantly, this nongenotoxic mode of tumorigenesis displays thresholds and differences in interspecies sensitivity, with higher primates being much more resistant than rodent species.

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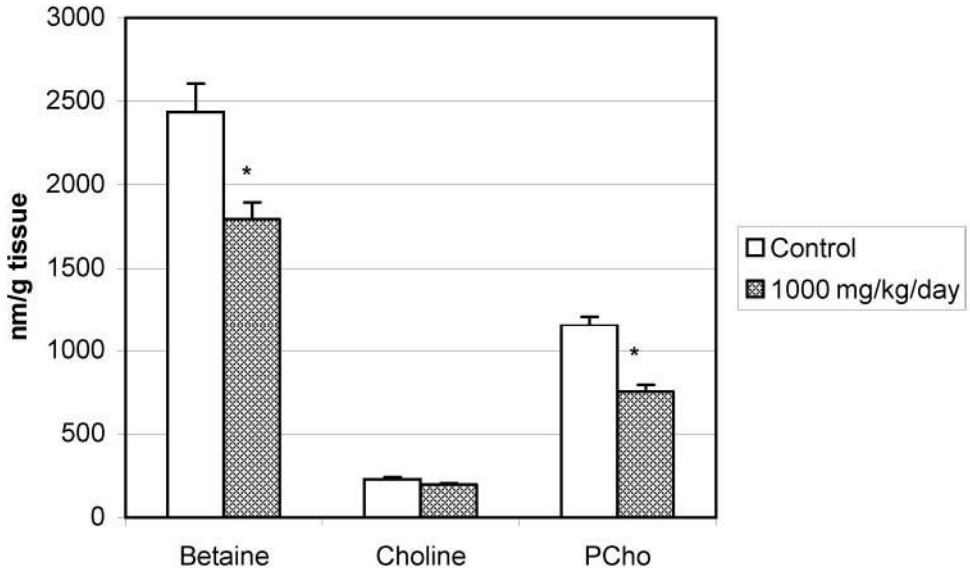

FIG. 1.

Levels of choline and metabolites in liver of female B6C3F1 mice administered 0 or 1000 mg/kg/day triethanolamine by dermal application for three weeks (average and standard deviation of 10 mice/group). * Denotes statistically identified as different from controls; Dunnett's Test, alpha = 0.05.

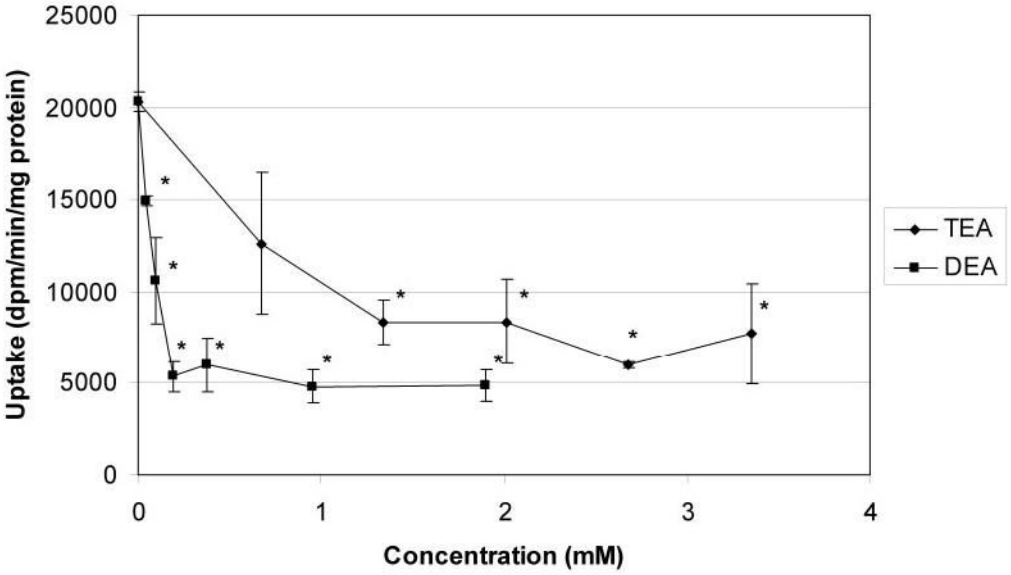

FIG. 2.

Uptake of ^{3}H-choline by Chinese Hamster Ovary Cells *in vitro* in the presence of neutralized triethanolamine or diethanolamine. ^{3}H-Choline was added to nonconfluent cultures for 10 min; cells were washed, harvested, and radioactivity determined using liquid scintillation counting (average and standard deviation of triplicate determinations). * Denotes statistically identified as different from controls; Dunnett's Test, alpha = 0.05.

TABLE 1

Levels of Choline and Metabolites in Liver of Female B6C3F1 Mice and CDF Rats Administered Triethanolamine by Dermal Application for Three Weeks

	Betaine (nmol/gram tissue)	Choline (nmol/gram tissue)	Phosphocholine (nmol/gram tissue)
B6C3F1 mice			
Control (acetone)	2911.1 ± 508.9	401.5 ± 178.6	1170.4 ± 191.5
10 mg/kg/day	2292.9 ± 374.2	525.9 ± 148.1	1193.3 ± 264.5
100 mg/kg/day	2574.9 ± 545.7	315.7 ± 121.1	956.2 ± 164.4
300 mg/kg/day	2412.1 ± 433.9	427.4 ± 121.6	942.6 ± 261.8
1000 mg/kg/day	2072.4 ± 241.6*	261.0 ± 67.1	679.2 ± 112.9*
CDF Rats			
Control (acetone)	3693.8 ± 493.2	113.7 ± 17.7	3400.5 ± 600.1
250 mg/kg/day	3569.4 ± 501.5	125.7 ± 21.0	3264.2 ± 274.7

Note: Analyses were by GC-mass spectroscopy as referenced in text (average and standard deviation of 8 animals/group; Trend Test *p*-values were 0.0029 for betaine, 0.0149 for choline, 0.0001 for phosphocholine.

*Statistically identified as different from controls, Dunnett's Test, alpha = 0.05.