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DOSE-RESPONSE STUDIES OF GENOTOXIC RODENT CARCINOGENS: THRESHOLDS, HOCKEY STICKS, HORMESIS OR STRAIGHT LINES?

This is a question posed in a recent article in the journal Toxicology by Kirk Kitchin and Janice Brown, U.S. EPA scientists, who observed less liver DNA damage than control groups at very low doses for a number of genotoxic carcinogens.

The following narrative represents a summary of the findings of Kitchin and Brown. Because the issue of deviations from linearity at low dosages is a central topic that the BELLE initiative is exploring as well as the dominating role that cancer risk assessment plays in environmental policy, it was felt that the Kitchin and Brown findings and interpretation should be explored by experts in the areas of study design/statistical analysis, carcinogen mechanisms, and risk assessment. Consequently, the BELLE office solicited independent commentary from a range of experts. These commentaries are presented in this issue along with a response from Kitchin and Brown who were sent the expert reviewer commentaries. We hope that this dialogue will prove intellectually stimulating as well as practically helpful to researchers and risk assessors who confront similar findings in their work.

The BELLE office invites the readership to submit written critiques/commentary responses to the information/questions debated in this issue of the newsletter. If significant new explanations, analyses, interpretation or data are provided, selected submissions will be considered for publication in a subsequent issue.

SUMMARY OF THE KITCHEN AND BROWN PAPER

A critical question faced by experimental researchers and risk assessors is how to interpret apparent deviations from linearity in the low dose range of toxicological/epidemiological studies especially those believed to be genotoxic carcinogens. A report by Kitchin and Brown entitled "Dose-response Relationship for Rat Liver DNA Damage Caused by 49 Rodent Carcinogens" (Toxicology 88:31-49, 1994) experimentally assessed DNA damage in female Sprague-Dawley rat liver in an effort to obtain insight on the nature of dose-response curves for chemical

carcinogenesis. DNA damage was selected as the measurement endpoint since all agents observed to damage hepatic DNA were also rodent carcinogens. Dose response curves for rat hepatic DNA damage were reported over an unusually wide dose range of up to six orders of magnitude. With limited exceptions, the lower doses selected were usually 1/10, 1/100, 1/1000, or 1/10,000 of the initial dose (i.e. usually 1/5 of the LD_{50}). Of the 49 rodent liver carcinogens initially selected for study, 12 were found to produce DNA damage [1,2-dibromoethane, 1,2-dibromo-3-chloropropane, 1,2-dichloroethane, 1,4-dioxane, methylene chloride, auramine O, Michler's ketone, selenium sulfide, 1,3-dichloropropene, 1,2 dimethylhydrazine, N-nitroso-piperidine and butylated hydroxytoluene (BHT)]. Eleven of the dose-response curves (i.e. with the exception of BHT) fit a linear model well ($r^2 = 0.886$) but a quadratic model better ($r^2 = 0.947$). Of the eleven chemical data sets, the quadratic regression analysis yielded a negative linear slope for all agents when plotted against logged but not unlogged dose. The authors concluded that the data "happened to have random variation around the control values which make some treated values lower than control values and causes the overall logged regression curve (Figure 5) to dip below zero at low doses". They further state the shape of the dose response curve was "exactly the opposite of what would be predicted on the basis of biochemical and pharmacological theories based on the law of mass action". Because of the simplicity of the linear model with no y-axis intercept and the fairly high r^2 of 0.886 for eleven different DNA damaging chemicals, the authors favor the simpler linear regression model using untransformed dose. Kitchin and Brown concluded that they do not believe that extremely low doses of chemical carcinogens actually decrease the degree of DNA damage found in treated animals or improve the animals health in anyway. Figures 1 #38; 2 provide the dose-response curves for the 11 agents. For those desiring to reconstruct dose-response curves for individual chemicals, figures 3 and 4 provide an individualized representation of two of the 11 agents without the co-presence of multiple dose response curves as presented in Figures 1 #38; 2. Figure 5 provides the modeled dose-response relationship for damage caused by an "average" DNA-damaging carcinogen.

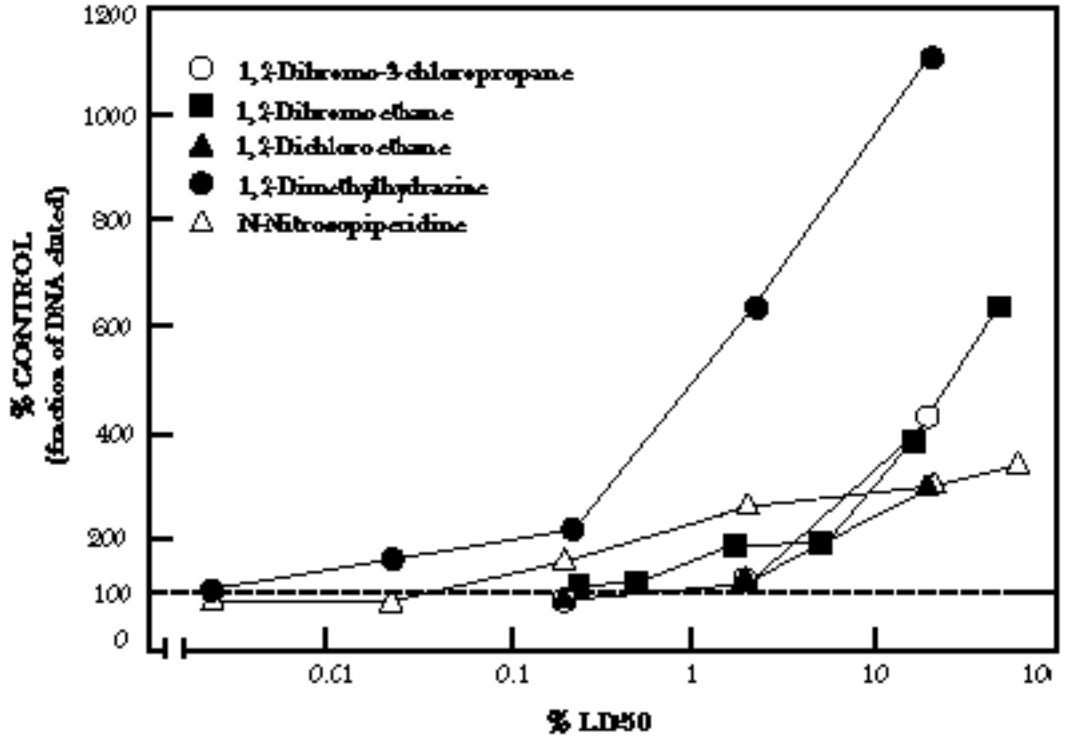

Figure 1. Dose-response relationship between DNA damage (the fraction of the DNA eluted expressed as a percent of control DNA) and the log of the dose of five rodent carcinogens of high potency in damaging DNA. The x-axis is calibrated in terms of the percent of each individual compound's LD50 (dose which kills 50% of the experimental animals)(NIOSH, 1976). Hepatic DNA was obtained from female rats given the various chemicals orally.

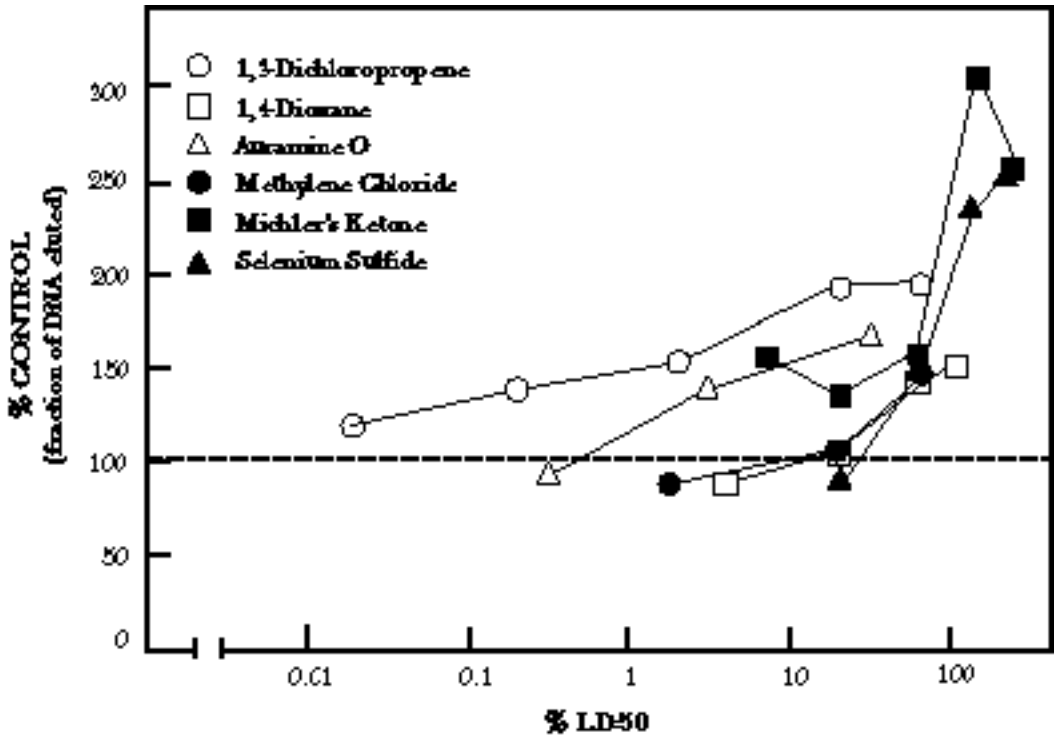

Figure 2. Dose-response relationship between DNA damage (the fraction of the DNA eluted expressed as a percent of control DNA) and the log of the dose of 6 rodent carcinogens of low to medium potency in damaging DNA. The x-axis is calibrated in terms of the percent of each individual compound's published LD_{50} (the dose which kills 50% of the experimental animals). Hepatic DNA was obtained from 5-15 female rats given the various chemicals orally.

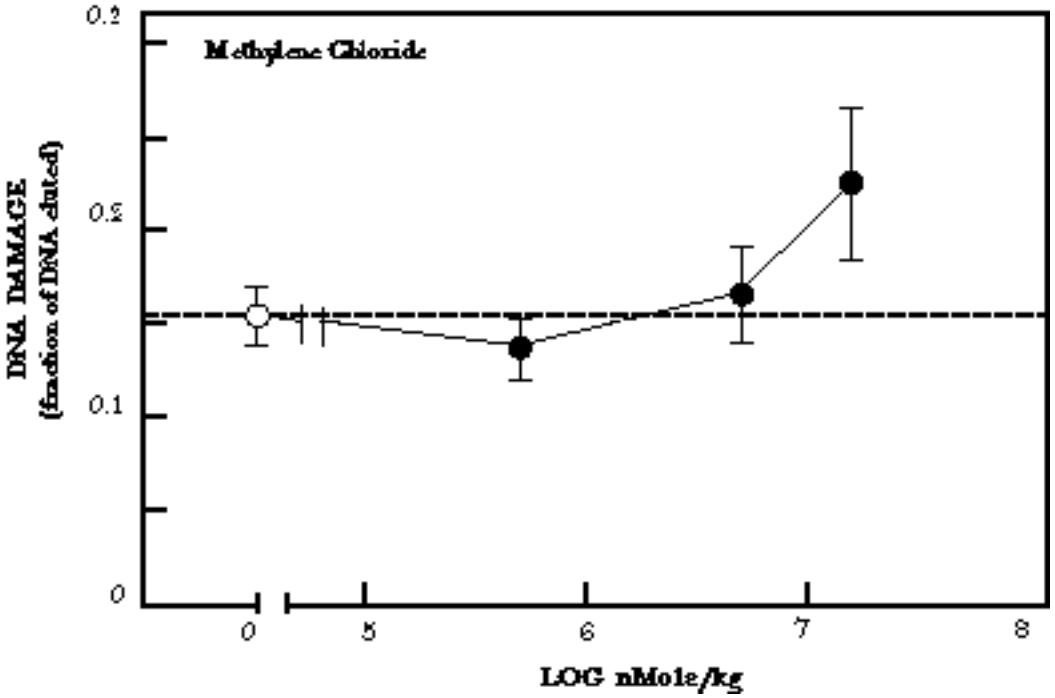

Figure 3. Dose-response relationship between DNA damage (the fraction of the DNA eluted) and the log of the dose of methylene chloride. Hepatic DNA was obtained from 8-15 female rats given methylene chloride orally.

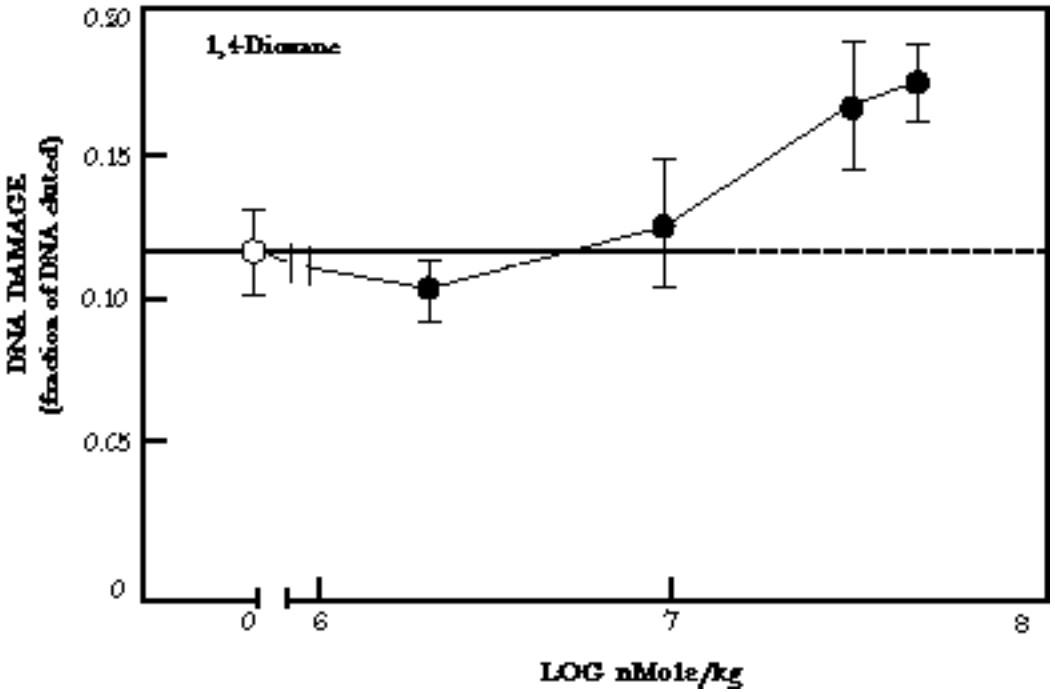

Figure 4. Dose-response relationship between DNA damage (the fraction of the DNA eluted) and the log of the dose of 1,4-dioxane. Hepatic DNA was obtained from 8-15 female rats given 1,4-dioxane orally.

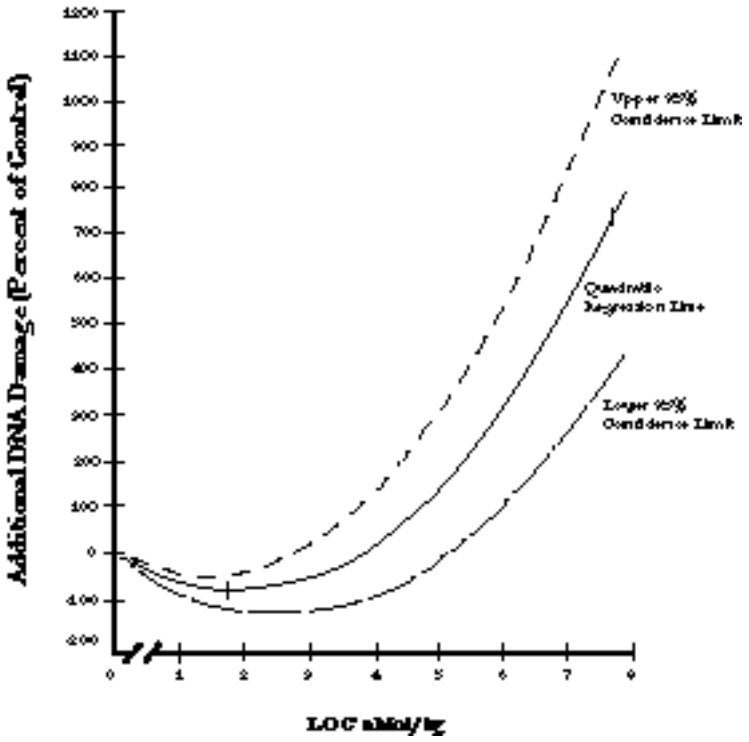

Figure 5. Dose-response relationship for female rat hepatic DNA damage caused by an "average DNA-damaging carcinogen". The mean of linear slopes and quadratic slopes of 11 chemical carcinogens were used to determine the quadratic regression line and the 95% confidence limits. On the central regression line, (‹‹‹), is the experimental dose range of this study (6 orders of magnitude); the highest 1,4-dioxane dose tested (4200 mg/kg, 7.68 on the log nmol/kg dose scale) is extremely close to the maximal achievable dose for animal experimentation; the lowest N-nitrosopiperidine dose tested was 0.004

mg/kg (1.55 on the log nmol/kg dose scale). The dashed portions of the central regression line, () represent extrapolations both above and below the experimentally tested dose range.

The Kitchin #38; Brown paper raises important issues of toxicological and possible public health significance. A brief listing of some of these issues is given below. The subsequent commentaries address some of these issues while raising new insights and challenges on their own.

Selected Issues:

1. Can the shape of the dose-response curve for DNA damage be useful for estimating cancer risk?
2. What is the predictive relationship between the low doses used in the genetic toxicology study of Kitchin #38; Brown and the higher doses employed in cancer bioassays.
3. What is the biological significance of using log transformed versus non-transformed data for interpretation purposes?
4. For each chemical displaying a U-shaped dose-response curve only one data point was observed to show an "apparent" protective effect. If indeed an hormetic (or protective) dose range has been entered what is the dose response of the hormetic range?
5. Even though only one dose was observed as "protective" per experiment and the quadratic model may not be statistically significant, does the observation that 8 of 11 agents display a "protective" response affect a weight of evidence judgement on the nature of the dose response

relationship?

6. If the quadratic model is preferred, what is the underlying biological/toxicological basis accounting for its shape?
7. Do the dose response curves really reflect genotoxic damage caused by these chemicals? Or is the assay being used actually measuring something else? In other words, is the apparent hormetic effect really a non-effect or cell killing (necrosis or apoptosis) at the low dose levels and death-related non-specific DNA degradation at high doses?

Comments on the Kitchen and Brown Paper

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AUTHOR'S APPROACH

Kitchin and Brown in their figure 14 (figure 5 in this paper) show the upper 95% confidence limits on the quadratic is lower than control in the low dose region. At face value, this indicates that the average DNA damaging carcinogen has a U-shape response function. Their method of analysis leading to this conclusion has some weaknesses.

Here's what was done. For each chemical, a quadratic was fit between the response (expressed as the difference from percent of control) and the log of the dose. The curve and the confidence limits were constrained to pass through the point where the log dose is zero. The plot is based on the averages of the 2 regression coefficients. The authors did not indicate the formula used for the confidence intervals, so the confidence intervals are probably the usual type that only indicate for a given dose how the corresponding point on the regression line may vary. In order to infer that the quadratic is significantly lower than control, one needs confidence intervals of the Working Hotelling-Scheffè type (Spurrier, 1988) that are about 43% wider (measured from the regression line) than those in figure 14.

Weaknesses With Author's Approach

Too much faith seems to be put into the quadratic model. While the quadratic model has a use in biological investigations, it lacks biological plausibility as a dose-response model. Therefore, its role should be restricted to approximation. For example, it could be used for evaluating features of the dose-response curve over a limited range of doses. Over broader ranges, the approximation might miss or distort features of the dose-response curve. In an extrapolation role, it must be used very cautiously.

The quadratic was not fit appropriately. It is unnecessary and particularly inappropriate to use a no y-axis intercept when studying the low dose region. If one keeps in mind that the zero dose is at minus infinity on a log dose scale and that the quadratic is only good within the range of the data, then there is no justification for the no y-axis intercept. Also, the conversion of the response to percent of control data does not by itself justify using zero-width confidence intervals even at the control dose.

The use of the average of the coefficients of the quadratic across chemicals is suspect. How was between chemical variation treated? The answer would have been apparent had the authors indicated the formula used for generating the confidence intervals.

One needs to keep in mind the null hypothesis when using the quadratic to detect U-shapeness. For carcinogens, the null hypothesis could be linearity with dose. For noncarcinogens, there is the threshold concept. In this case, a simple model is the hockeystick model which is flat up to the threshold and then linear with dose or log dose above the threshold. To challenge the status quo in both situations, one could adopt the hockeystick as the null hypothesis and attempt to show that the evidence better supports the U-shape. However, this approach is not going to be totally convincing if there are few doses in the low dose range. For example, an apparent U-shape quadratic can result from the data exactly fitting the hockeystick.

An Alternative Approach

A meta-analysis was done of data kindly provided by Dr. Kitchin. This data was in the form of means and standard deviations. We will (1) show how meta-analysis deals with some of the issues that were just mentioned, (2) show the results of the meta-analysis, and (3) discuss those results.

Issues addressed by the Meta-Analysis

None of the individual chemicals had a response mean significantly lower than the control response. Yet, meta-analysis offers the possibility of using data from all the chemicals to test the hypothesis that the average DNA damaging carcinogen has a U-shape dose-response function. A new method of using meta-analysis based on hierarchical linear models developed by Raudenbush and Bryk (1985) and DuMouchel (1994) permits testing the hypothesis even when the indicator of U-shapeness varies in the target population.

For each dose, the effect size (ES), sometimes better known as Z-score, is the ratio of the dosed mean response minus the control mean response to the pooled standard deviation. The standard deviation of the ES was calculated following Raudenbush and Bryk (1985) as the square root of $(n_c+n_e)/n_cn_e + ES^2/(2*(n_c+n_e))$ where n_c and n_e are the sample sizes at the control and exposed dose, respectively.

A minimum effect size indicating the U-shapeness for each chemical was calculated using one of two methods ‹ the descriptive method and the quadratic method. We wanted to use the best of these two methods because we were not sure which would provide a better indicator of U-shapeness. For example, if there are many doses near the minimum then the quadratic method might be better. Also, the descriptive method suffers from certain types of multiple inference errors.

Rubin (1992) in a thought provoking article considers two approaches toward meta-analysis. One is a literature synthesis which had the summarization of the literature as its goal. The second is an attempt to estimate effect size surfaces so that the noise has been reduced to the extent possible. Our descriptive method has the qualities of literature synthesis, while our quadratic method may yield an indicator more sensitive to the hypothesis.

The descriptive method was:
The smallest of the effect sizes for each chemical was used as an indicator of U-shapeness.

With this effect size one needs an indication of toxicity because in the toxic region there is no chance of detecting a U-shape dose-response. Cohen (1988) has defined 0.5 as a medium effect size. Therefore, if the effect size exceeded 0.5, it was viewed as an indication of toxicity, and the ES at the dose was omitted from the analysis in order to address the masking effect of toxicity.

Next, an adjustment for multiple comparison was made to address the issue that when more nontoxic doses are assayed there is an enhanced possibility that a mean lower than the control mean is attributable to chance. The adjustment was accomplished with the use of a table from Dunnett (1955) one-sided comparisons. For chemicals having m nontoxic doses, the standard deviation of the effect size was multiplied by the ratio of the critical t for m comparison divided by the critical t for 1 comparison.

The quadratic method was:
Minimum effect sizes were estimated using the minimum of the quadratic fit by least squares regression on the log of the three lowest (nonzero) doses. A perfect fit is possible, and the variance of the unexplained error was replaced by the average of the variances of the three effect sizes used in the regression. The variances of the estimated extreme effect sizes were estimated using the Working-Hotelling-Scheffè method (Spurrier, 1988). This leads to confidence intervals of the type that allow inferences to be made for all log doses, and not just for a single given dose. This modification was introduced by multiplying the usual variance estimates by the factor $p*F_{p,N-p,0.095}/F_{1,N-p,0.975}$ where p is the number of parameters in the regression model (p=3 for the quadratic model) and N-p is the total degrees of freedom of the effect sizes used in the regression.

Next, the best of these two estimates of minimum effect size was selected by choosing the effect size with the smallest coefficient of variation, and these best estimates were used as the effect size variable in the meta-analysis.

Results of the Meta-analysis
Three of the 12 regressions had negative quadratic coefficients indicating that the extreme of the fitted quadratic was a maximum. Therefore, effect sizes estimated for these three chemicals based on the quadratic method were ignored. Effect sizes were based on the quadratic method for 2 of the chemicals.

Table 1 shows the effect sizes, the variance of the effect sizes, and the number of doses when the effect size was less than 0.5.

Table 1.			

Chemical	Number of nontoxic doses	Minimum Effect Size	Standard Error of ES
1,2-Dibromo-3 chloropropane	1	-0.714	0.621
1,2-Dibromoethane	2	0.227	0.479
1,2-Dichloroethane	1	-0.281	0.451
1,2 Dimethylhydrazine	1	0.172	0.578
1,3-Dichloropropene	1	0.311	0.474
1,4-Dioxane	2	-0.326	0.551
Auramine O	1	-0.195	0.457
Butylated Hydroxytoluene	2	-0.799	0.733
Methylene Chloride	2	-0.376	0.489
Michler's Ketone	0		
N-Nitrosopiperidine	2	-1.209	0.886
Selenium Sulfide	1	-0.233	0.501

One nontoxic effect size was deleted because the presence of lower toxic doses suggested that the small magnitude of this effect size was due to high dose reversal. Only one or two doses were nontoxic for each chemical. The data for Michler's Ketone was deleted because all the doses were toxic by this standard.

Meta-analysis (Raudenbush and Bryk, 1985) of table 1 data indicated that there was no significantly negative effect. The between chemical variance was not significantly different from zero, and the upper limit of the 95% confidence

interval on the variance was 0.1. The estimated overall effect size was -0.209. A power analysis indicated an assay of 51 DNA damaging chemical carcinogens should detect such an effect size at the 5% one-sided significance level and 80% power.

Discussion

The meta-analysis suggests that there is not enough evidence presented in the article to conclude that typical DNA damaging carcinogens are U-shaped. This is rather surprising since the average minimum effect size corresponded to a departure of 8% below the control mean. This is a rather large effect since 5% was used as the definition of U-shape in a survey of the literature (Davis and Svendsgaard, 1994).

If the dose-response curves for all the chemicals were U-shaped, identical, and the U-shape curve is peaked, some variation in the minimum effect size between chemicals would be expected due to the doses varying in location relative to the dose corresponding to the minimum effect size. Nevertheless, finding that the 'between' chemical variance was not significantly different from zero is not an indication that the curves are not U-shaped, since it could also not be ruled out that this variance could be quite large. Thus, more data are needed.

Statistical significance of apparent U-shapes was not obtained because the method of choosing doses for these 12 chemical carcinogens resulted in usually only one and never more than two doses in the low dose region. One needs more doses when relying on the pattern of the dose-response to determine the shape and to detect U-shapeness. Also, the sample size used in the assays was insufficient for detecting U-shapes of the usual magnitude encountered in the literature, while sufficient for the detection of strong DNA damaging chemicals. Vehicle or other explanatory factors may also play a role.

At this stage in the investigation of U-shape curves an exploratory mode is required. One cannot reject hypotheses based on unsuitable data. At the same time, one needs to be aware of appropriate methods for confirming U-shape curves, and exploratory methods for teasing out useful hypotheses for later use in confirmatory testing.

REFERENCES

Cohen, J. (1988). Statistical Power for the Behavioral Sciences, Lawrence Erlbaum Associates, Publishers,

Hillsdale, New Jersey.

Rubin, D.B. (1992). "Meta-analysis: Literature Synthesis or Effect-size Surface Estimation",Journal of Educational Statistics 17:363-374.

Davis, J.M. and Svendsgaard, D.J. (1994). "Nonmonotonic Dose-response Relationships in Toxicological Studies", in Biological Effects of Low Level Exposures: Dose-response Relationships Edited by E.J. Calabrese, Lewis Publishers, Boca Raton.

DuMouchel, W. (1994). "Hierarchical Bayes Linear Models for Meta-Analysis", Unpublished Manuscript.

Dunnett, C.W.(1955). J. Am. Stat. Assoc.50:1115-16.

Raudenbush, S.W. and Bryk, A.S. (1985). "Empirical Bayes Meta-Analysis", Journal of Educational Statistics, 10:75-98.

Spurrier, J.D. (1988). "Working-Hotelling-Scheffè Confidence Bands" in Encyclopedia of Statistical Sciences, Vol. 9. p. 651-653. J. Wiley #38; Sons.

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The numbers of animals used in this study is very small. The numbers are so small that I suspect that almost any curve would fit them. When the linear and quadratic both fit equally well it usually is an indication that the sample is

too small and it appears suspiciously that this is likely the case here.

The shape of the curves when viewed visually do not appear that unusual. Likely if plotted on linear scales they would look like straight lines increasing as the dose increases. The vertical scale is usually the probability of an effect and conversion may change the shape of the curve.

The idea of the effect of a possible threshold is only mentioned in the paper. Did the authors consider the effect of the presence of a threshold would have on their analysis?

Finally, it would be most helpful if the confidence limits on the r squared test were computed. For example, the two values quoted in the abstract of 0.886 and 0.947 are probably really the same value, within the error bounds. Perhaps another better test of the goodness of fit could be used.

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In this study the authors used DNA damage in female rat liver, measured by alkaline elution techniques, as a surrogate for carcinogenesis (tumor formation) in constructing dose response curves. The basis for using this approach was that all chemicals used in the study were carcinogens, and had produced DNA damage in female rat liver. The dose response curves were constructed using the log of the molar dose as well as by using the per cent of the oral LD_{50} . The curves were analyzed by regression methods. They were found to fit a linear model and a better fit was found with a quadratic model. For the quadratic model, a negative linear slope was determined when the data was plotted against log dose, but not when plotted against non-logged dose. In essence, less damage was produced at the lower dosages, than in controls. The authors go on to say, they do not believe that carcinogens are beneficial to the animals at the lower dosages and that random variation of the control values is responsible for this curious effect.

Our attention has focused on the explanation given by the authors for the negative slope. They concluded that random variation in the controls may be responsible for the observation. After careful consideration of various

possibilities, we offer the following as an alternative explanation. First, it should be stated that the rate of DNA damage at the lower dosages, although less than control, is not statistically significant. This does not however, preclude these effects from being biologically significant. We believe the issue of repair of DNA damage is critical in possibly explaining these results at the lower dosages.

What we believe is being measured by the alkaline elution assay is the net result of DNA damage versus DNA repair, not merely DNA damage. Thus the negative quadratic coefficients could indicate a stimulation of DNA repair that exceeds the rate of DNA damage at low dosages. At higher dosages, the rate of DNA repair would be, ultimately, overwhelmed by the rate of DNA damage. The stimulation at lower levels induce repair enzymes, endonuclease, polymerase, ligase. A recent paper by Mehendale describes the temporal aspects of tissue repair and injury as a function of dose in liver tissue. It was pointed out that at low doses there is a dose related increase in stimulation of repair. He believes his results demonstrate that tissue repair and hepatic injury are two dynamic but opposing events occurring simultaneously in a critical time frame subsequent to injury. It appears to us, the same phenomenon is occurring with respect to DNA repair to carcinogen insult to liver cells.

Mehendale's observations focused primarily on the triggering of cell division as the means of repair. In the present studies, repair would be less visible and would occur in the DNA. Thus it would not be necessary for cell division to occur in order for the damage to be repaired. There have been numerous observations of lower levels of chemicals stimulating activity . One of the earliest reports of weak stimuli increasing physiological activity while very strong stimuli inhibit or abolish, was that of Smyth.

A similar issue has been the subject of a continuing controversy with regard to the biological effects of low level radiation exposures (radiation hormesis). S. Hattori described the current research efforts in Japan directed toward understanding the mechanisms of how cells and organisms adapt to environmental stimuli such as low dose radiation. D. Billen described some scientific findings regarding spontaneous DNA damage and its significance to understanding low dose radiation effects. He points out that enzymes involved in repair of DNA lesions are similar whether DNA damage is produced spontaneously or by radiation. However, radiation is known to induce an ERROR-PRONE REPAIR system in bacterial cells and perhaps in mammalian cells, as well. We thus believe there is an alternate explanation for the results at the lower levels of exposure seen by the authors, and that is stimulation of repair.

We do agree with the authors that lower levels of carcinogens can not be considered beneficial. Although the DNA damage is repaired, errors can and do occur in the process. Thus there would be cells with genetic damage, mutations, that would give rise to daughter cells with this damage. The consequences of this damage is difficult to predict for it would depend on the magnitude and significance of the mutations.

REFERENCES

Mehendale, H.M., Mechanism-based Predictions of dose-response relationships: Why low doses of CCl4 are non-toxic. BELLE Newsletter, Vol.2, No. 3. April, 1994.

Smyth, N.F. , Sufficient challenge, Food and Cosmetic Toxicology 5: 51-58, 1967

Hattori, S., State of research and perspective on radiation hormesis in Japan. BELLE Newsletter, Vol. 3, No.l, July 1994.

Billen, D., Spontaneous DNA damage and its significance for the negligible dose controversy in radiation protection. BELLE Newsletter, Vol.3, No.l, July 1994.

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This interesting paper attempts to address the "dose-response curves for chemical carcinogenesis". The underlying concept is really quite novel in that there is no a priori reason to believe that a chemical that induces, for example, tumors in B6C3F1 male mouse lung in an inhalation study, should produce alkali-labile sites and DNA strand breaks (the DNA damage referred to in the title) in female Sprague-Dawley rat liver by gavage. Due in part to differences in target organ, route of administration, and animal strain used, it is not surprising that the technique applied here had

little success in identifying "carcinogens". What remains however is interesting relative to its significance on other measurements performed in these experiments.

One example is the relationship of the doses tested in these studies to the LD50. Acute toxicity rarely results from liver toxicity, but instead usually results from either neural, cardiac, or respiratory damage. For instance, acute toxicity induced by carbon tetrachloride, a potent hepatotoxin, acts by depressing the central nervous system (1). Acute toxic mechanisms, especially at the doses used for these agents may alter normal physiology thereby obscuring changes that might be seen in the liver after chronic dosing with agents. This may in part account for the surprising result that for a number of the compounds given at three-fifths the LD50 dose, including the well-characterizing liver damaging agents aflatoxin B1 and carbon tetrachloride, negative effects are observed in this assay.

Focusing on the agents which induce "hepatic DNA damage" at doses less than one-fifth the LD50, and thus less likely to induce physiological abnormalities, it is interesting to note that these three compounds, 1,2-dibromoethane (DBE), N-Nitrosopiperidine (NNP), and 1,2-dimethylhydrazine (DMH) have the highest (and most significant) negative quadratic slopes in the regression analysis. If "hepatic DNA damage" reflects some form of hepatocellular damage, what these results may suggest is that a low dose of agent results in less liver damage than a control. There are many potential mechanisms for this. For DMH and NNP, one simple one is that deionized water, used in the zero dose gavage, is damaging to the liver and low doses of these agents (as salts) help balance the osmolality of the solution, resulting in less damage. DBE at doses of 1 mg/day interferes with sexual function in bulls (2), suggesting that there is a disruption of the hormonal control of gonadotrophin involved in its toxicity. If DBE acted as an agonist, it could have stimulatory effects at low doses, while disturbing the gonadotrophin axis at higher doses, all of which could impact on the hormonally sensitive liver. More complicated mechanisms include effects on nutritional factors (3), differential metabolism of these compounds at low doses, which in turn could modulate the effect of these agents on the liver, and different effects (e.g., saturation of metabolism) at higher dose with different consequences for liver, possible threshold stimulation of DNA repair, etc.

Given these evident possibilities, it is surprising that this paper so readily disavows the obvious consequences of its own data analysis, based on "the authors do not believe". Although few compounds are evaluated at doses not likely to be confounded by the effects of acute agent toxicity, the analysis concerning these few agents appears to lead to

significant results. It is likely to be more fruitful to explain results rather than explain them away.

REFERENCES

1. IARC, Carbon Tetrachloride. In: Some Halogenated Hydrocarbons. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 20., IARC Publications, Lyon, France, 1979, pg. 384.
2. Murphy, S. Pesticides. In: (L. Casserett, J.Doull, eds.) Toxicology, Macmillian, N.Y., 1975. ppg. 408-453.
3. Turturro, A. and Ronald Hart. Modulation of Toxicity by Diet: Implications for Response at Low-level Exposures. In: Biological Effects of Low Level Exposures: Dose Response Relationships (Edward Calabrese, Ed.) Lewis Publishers, Chelsea, MI 48118, 1994. ppg. 143-152.

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INTRODUCTION: GREAT DATA/BANKRUPT PARADIGM

In science, experimental data, no matter how well they are derived, are not self-evident in their interpretation. All experimental observations must be interpreted and the interpreter uses, among other things, the accepted prevailing paradigms (l). To their credit, Kitchin and Brown (2) have performed a valuable experiment and analysis that beg a critical examination of the implicit and explicit assumptions related to the major paradigm used in the design and interpretation of the data.

Fundamentally, in order to get at a very important scientific question, namely, "What is the nature of the dose response at low dose exposures of physical and chemical carcinogens?", Kitchin and Brown have measured the induction of alkaline-labile sites and single strand breaks induced by rodent carcinogens over a wide concentration range. Key to this evaluation of their data is that Kitchin and Brown have interpreted these data as an indication of

DNA damage, which will lead to mutations in the cell(s), giving rise to the cancers found in the animals treated with the chemicals being studied. What follows is a re-examination of the interpretation of short-term tests for genotoxicity by these chemicals, the use of bulk DNA from the treated target tissue as a true representative of the state of DNA in the few target cells in the target tissue which will lead to cancer, the various theories of carcinogenesis and the protocol from which are derived the rodent carcinogenicity data.

"CARCINOGEN AS MUTAGEN" AS THE PREVAILING PARADIGM IS BANKRUPT

Taken as a given will be all the data used in this report (e.g., rodent carcinogenicity data, LD50 information; alkaline labile data; purity of the chemicals; statistical analyses and choice of data transformation, etc.). However, what will be challenged is the assumption that a chemical is a rodent carcinogen because of its "genotoxicity" or DNA damaging/mutagenic activity as determined by short-term "genotoxicity" assays (3,4). In addition, an examination will be made of the assumptions related to the nature of carcinogenesis, the limitations of all short-term assays used to detect genotoxic chemicals, and the protocol to classify "carcinogens" in the long-term bioassay.

In the ABSTRACT of their paper, the authors have assumed that the 12 rodent carcinogens tested in their paper were genotoxicants because "Š all chemicals found to damage hepatic DNA were rodent carcinogens." While the authors do acknowledge that "Šwith many surrogate experimental parameters for cancer bioassays, interpretation of the results is limitedŠ," they, however, persist in not rigorously questioning the interpretation of data derived from these short term tests for genotoxicity or the cancer bioassay results. The crux of the challenge to the interpretation of the results of this paper, in this reviewer's opinion, is the idea that both the bioassay cancer data and the "genotoxicity" data from short-term tests do not purport to represent what most think they represent. Therefore, the use of these two very questionable data sets to validate one another is highly risky.

Carcinogenesis is now generally accepted as a multi-step, multi-mechanism process (5). In addition, it would be very difficult to deny that mutagens and mutations play a role in that process, given genetic, molecular oncological and direct DNA sequencing of critical oncogenes or tumor suppressor genes found in tumor cells (6). However, carcinogenesis is more than mutagenesis (7). Regardless of the number and sequence of the mutagenic changes that might occur in a given cancer, the point is that there is more than one alteration during carcinogenesis (8), and some of them involve non-mutagenic or epigenetic changes (9,10,11,12). When any chemical interacts with a cell, it will not just damage DNA. In fact, the first structural entity a chemical interacts with is the cell membrane. That

encounter, because of the nature of cell membranes, will trigger a wide range of receptor and non-receptor signal transduction mechanisms and membrane perturbations (e.g., changes in membrane fluidity; ion channel activation; enzyme activation, etc.). Could it be possible that at low doses, physical and chemical agents alter gene expression (transcriptionally, translationally, or posttrans-lationally) (13-15). At higher doses, those associated with some cell killing, release of nucleases from destroyed membranes could lead to apparent DNA breaks as they are in the process of dying (16,17). In addition, in vitro or in vivo, where DNA-related parameters are being measured, the death of cells release all kinds of products which could act to induce epigenetic responses in surviving cells (e.g., stimulate wound healing, and compensatory cell proliferation).

One can now argue, "What of the lesions measured in DNA of target tissue exposed to chemical and physical agents which bring about cancers in rodents? When DNA from a target organ is extracted after an animal is exposed to a chemical which brings about a cancer, it is frequently loaded with measurable lesions (e.g., 32P -post -labeling, chemical detection of specific DNA base damages, etc.). It is normally assumed that this constitutes proof that any mutations found in given oncogenes/tumor suppressor genes in the tumors of these treated animals were induced as the result of the DNA lesions measured after the exposure of the chemical or physical agent.

If one assumes that all cells of the tissue are equal "targets" for carcinogenesis, then this argument might be plausible. However, if one assumes (which few in the field of carcinogenesis do) that there are only a few cells that are target cells for carcinogenesis [The stem cell theory (18)] (19), then bulk DNA analyses of tissue would not necessarily reflect the status of DNA of the few stem cells of this tissueŠStem cells, by definition, are less differentiated than their daughter differentiated progeny. Therefore, they might be expected to be different in their ability to metabolize chemicals, protect and repair their DNA. In any given tissue, the numbers of differentiated progeny would be expected to be more numerous than the stem cells (20). When DNA is extracted from tissues exposed to physical or chemical agents, DNA from the differentiated cells would swamp the DNA from the few stem cells. If there is a difference in metabolism, DNA damage and repair between the stem and differentiated progeny, and if the stem cells are the target cells for carcinogenesis, bulk DNA patterns of "lesions" would not be a marker for the chemical's carcinogenic mechanism of action (21).

Recently, Cha et al. (22) have shown that mutations in Hras oncogenes in mammary tumors of rats exposed to N-nitroso-N-methylurea arose from preexisting ras mutants in the tissue and that "an independent effect of NMU was

directly or indirectly responsible for tumor formation." Several other reports have obtained results in which a presumptive mutagen (based on some positive result in one short-term genotoxicity assay) appeared to act more as a promoter than an initiator; that is they acted as a mitogen rather than a mutagen (23-25).

Short-term tests (e.g., unscheduled DNA synthesis; drug-resistance markers as surrogates for DNA mutations, such as 6-thioguanine resistance, thymidine kinase deficiency, diphtheria toxin resistance; sister chromatid exchanges; 32P postlabeling; DNA alkaline lability; micro-nuclei; comet assay; etc.) all have potential artifacts, which, all too often, are not considered or controlled in the interpretation of the results. For example, in principle, while true mutagens and DNA damaging agents could alter the UDS, SCE, alkaline lability, and drug resistance markers, chemicals could induce UDS, SCE's, drug resistance, alkaline lability, etc, by non-DNA damaging mechanisms. For example, if a chemical could transcriptionally repress the TK or HGPRT genes, one could end up with cells which are TK- and 6-thioguanine resistant. These cells do not have mutated TK or HGPRT genes. Okadaic acid, a phosphatase inhibitor, was interpreted to be a powerful mutagen, using the diphtheria toxin marker as a measure of "genotoxicity" (26). However, it was not a mutagen in other short-term tests for genotoxicity. An alterative interpretation of these data would be that since diphtheria toxin resistance phenotype is based on the functionality of the EF-2 protein, phosphorylation changes in the protein due to the okadaic acid might alter the structure/function of the protein to bind with the diphtheria toxin. Therefore, okadiac acid would be conferring resistance by an epigenetic, not mutagenic, mechanism.

CARCINOGENESIS IS MORE THAN MUTAGENESIS: SOME MUTAGENS AND NON MUTAGENS ARE CARCINOGENS VIA EPIGENETIC MECHANISMS

The initiation phase of carcinogenesis appears to involve an irreversible process that prevents the "mortalization" or terminal differentiation of a stem cell (27,28), which is, by definition, immortal. This could be the result of a mutagenic or stable epigenetic process. However, if the conversion of the single initiated cell to a metastatic cell involves two or more genetic and epigenetic changes, the probability of all these changes occurring in that single cell would be the product of all the independent probabilities (29,30). Since the product of multiple rare critical events occurring in this single cell is exceedingly small, cancers would rarely occur. However, the promotion process of carcinogenesis involves the clonal amplification of the initiated or terminally-resistant cell (31). In effect, promotion is the process by which that single initiated cell with one critical event can be increased so as to increase the "target size" for the second hit. Further clonal amplification of the cell with two hits can increase the probability that additional hits

needed to transform the cell neoplastically would occur (29,30). Agents, which can increase the number of initiated cells by stimulating mitogenesis by wound healing, compensatory hyperplasia due to necrosis or by non cytotoxic mitogenic means, would be promoters rather than initiators. Additionally, agents, which might block apoptosis, could prevent the removal and therefore, allow the increase of initiated cells (32,33). Chemicals which are mutagens can also kill cells and therefore, at cytotoxic levels, be indirect promoters by stimulating surviving initiated cells (30). There are chemicals which are either non-mutagens for any cell or are mutagenic at high concentrations in cells which metabolize them. These chemicals would only be promoters at cytotoxic doses. Thresholds would probably characterize their action as promoters (34-38). Lastly, there are chemicals which are classic tumor promoters, which are neither mutagenic or cytotoxic at the levels they can clonally expand initiated cells [Phenobarbital, saccharin, PBB's, DDT, etc.] (34-38). These kinds of chemicals have been associated with thresholds in vivo during their action as promoters, as well as in vitro assays to measure inhibition of gap junctional communication, a postulated mechanism of tumor promotion (39). In these cases, long-term treatment of animals not previous experimentally treated with a known initiator can also produce a few tumors. One interpretation is that these promoters are "weak mutagens." Another explanation is that these non-mutagenic chemicals have selectively promoted spontaneously-initiated cells.

Another recent "fad" idea in the field of toxicology and carcinogenesis is that agents, which are known to generate reactive oxygen species (ROS) and are associated with various toxicology endpoints, bring about their effects because of oxidative damage to DNA. With all the chemicals which act as tumor promoters and also generate ROS but do not act as initiators, one has a hard time reconciling that DNA is the primary target of these ROS's. Recently, de Kok et al. (40), using linoleic acid to generate superoxide anion, did not find DNA damage in cells treated with this class of polyunsaturated fatty acids. Earlier, Alysworth et al. (41), showed that this class of polyunsaturated fatty acids inhibited gap junctional intercellular communication, a membrane related function. Moreover, Chang et al. (42), showed that other chemicals, such as cholesterol epoxides, suspected of being a genotoxic carcinogen, did not induce ouabain resistant mutations in Chinese hamster V79 cells. On the other hand, these compounds inhibited gap junctional intercellular communication. Therefore, the most likely targets for these chemical toxicant generated ROS are not DNA molecules. In effect, the logic, intrinsic to the "carcinogen as mutagen" paradigm, leads to the idea that because ROS are generated by chemical toxicants, the target must be DNA. The fact that free radicals can be generated by ionizing radiation and cause DNA damage does not mean that chemical induction of ROS will also damage DNA. The major difference is the direct deposition of ionizing radiation energy near the DNA, whereas the

chemical must first enter the cell through the cell membrane. The DNA of a cell is protected by many defense mechanisms to chemical generated ROS, whereas the cell membrane would be more susceptible. Therefore, the evidence suggests chemical-induced oxidative damage might be more of a epigenetic agent that a mutagenic one.

All of this is to highlight the observation made by Kitchin and Brown that the "mutagenic carcinogens," used in their studies, all had "thresholds" in their ability to induce alkaline labile DNA breaks. Might this have been due to the level of each compound to induce membrane triggered events in those cells in which either cell death was induced or epigenetic changes, such as altered DNA methylation occurred which might render the DNA labile to the assay treatment?

It was surprising that the original 12 chemicals used in this study included butylated hydroxytoluene as a DNA-damaging rodent carcinogen. Butylated hydroxytoluene is generally regarded as a non-genotoxic, tumor promoter (43,44). Other "carcinogens," normally judged as genotoxic carcinogens have also been shown to act as promoters [e.g., cigarette smoke condensates; 2-acetylaminofluorene] (45,46).

CONCLUSIONS

In summary, the authors' interpretation of these data seems to have excluded many important factors which probably are needed for a more biologically-relevant explanation of the experimental data: for example; (a) the possibility that only a few stem cells are the targets for carcinogenesis; (b) "positives" in short-term tests for genotoxicity can be misinterpreted epigenetic events; (c) DNA lesions measured in bulk tissue of exposed target organs might not reflect molecular events in the few target (stem cells) for the carcinogenic process; (d) mutations measured in oncogenes and tumor suppressor genes in tumors found in animals exposed to physical or chemical carcinogens might not be the result of mutations induced by the chemicals; (e) cytotoxicity (necrosis) of a carcinogen could act as a tumor promoter by stimulating compensatory hyperplasia. Alternatively, apoptosis could remove initiated cells. Some chemicals, by blocking apoptosis, could actually contribute to promotion. In both cytotoxicity mechanisms, the contribution of the chemical would be by mitogenesis or clonal expansion, not by mutagenesis; and (f) also since many non-genotoxic carcinogens can be directly mitogenic without being cytotoxic, these chemicals might be selecting out pre-existing spontaneously initiated stem cells. Many, if not all, promoters, work above threshold levels. Therefore, to this reviewer, it was no surprise that the results obtained appeared "exactly opposite ofŠpredictedŠ" The reason is because "carcinogen as mutagen" is not the paradigm I would use to interpret short-term tests for

genotoxicity or long term bioassay rodent data.

REFERENCES

1. Kuhn, T.S. (1962). The structure of scientific revolutions, University of Chicago Press, Chicago.
2. Kitchin, K.T. and Brown, J.L. (1994). Dose-response relationship for rat liver DNA damage caused by 49 rodent carcinogens. Toxicol. 88:31-49.
3. Trosko, J.E. (1984). A new paradigm is needed in toxicology evaluation. Environ. Mutagen. 6:767-769.
4. Trosko, J.E. (1988). A failed paradigm: Carcinogenesis is more than mutagenesis. Mutagen. 3:363-364.
5. Weinstein, I.B., Gattoni-Celli, S., Kirschmeir, P., Lambert, M., Hsiao, W., Backer, J. and Jeffrey, A. (1984). Multistage carcinogenesis involves multiple genes and multiple mechanisms. J. Cellul. Physiol. 3:127-137.
6. Bishop, J.M. (1991). Molecular themes in oncogenesis. Cell 64:235-248.
7. Trosko, J.E. (1994). Radiation-induced carcinogenesis: Paradigm considerations. In: Biological Effects of Low Level Exposures: Dose Response Relationships. Calabrese, E.J. (ed.). Lewis Publishers. Boca Raton, FL, pp. 205-241.
8. Vogelstein, B., Fearon, E.R., White Smits, A.M.M. and Bos, J.L. (1988). Genetic alterations during colorectal tumor development. New Engl. J. Med. 319:525-532.
9. Kerbel, R.S., Frost, P., Liteplo, R., Carlow, D.A., and Elliott, B.E. (1984). Possible epigenetic mechanisms of

tumor progression: Induction of high-frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cell populations by 5-azacytidine treatment. J. Cell Physiol. 3:87-97.

10. Weinstein, I.B. (1991). Nonmutagenic mechanisms in carcinogenesis: Role of protein kinase C in signal transduction and growth control. Environ. Health Perspect. 93:175 179.
11. Holliday, R. (1987). DNA methylation and epigenetic defects in carcinogenesis. Mutat. Res. 181:215-217.
12. Rubin, H. (1994). Cellular epigenetics: Control of the size, shape, and spatial distribution of transformed foci by interactions between the transformed and nontransformed cells. Proc. Natl. Acad. Sci. USA 91:1039-1043.
13. Boothman, D.A., Lee, S., Trask, D.K., Dou, Q.P., and Hughes, E.N. (1990). X-ray induced proteins and genes in human cells. In: Ionizing Radiation Damage to DNA. Wallace, S. and Painter, R., (eds.). Wiley-Liss, New York, pp. 309-317.
14. Woloschak, G.E., Chang-Lia, C.M., Panozzo, J. and Libertin, C.R. (1994). Low doses of neutrons induce changes in gene expression. Radial. Res. 138: S56-S59.
15. Keyse, S.M. (1993). The induction of gene expression in mammalian cells by radiation. Semin. Cancer Biol. 4:119-128.
16. Bradley, M.O., Taylor, V.I., Armstrong, M.J. and Galloway, S.M. (1987). Relationships among cytotoxicity, lysosomal breakdown, chromosome aberrations, and DNA double

strand breaks. Mutat. Res. 189:69-79.

17. Galloway, S.M., Deasy, D.A., Bean, G.L., Kraynak, A.R., Armstrong, M.J. and Bradley, M.O. (1987). Effects of high osmotic strength on chromosome aberrations, sister chromatial exchanges and DNA strand breaks and the relation to toxicity. Mutat. Res. 189:15-25.
18. Trosko, J.E., and Chang, C.C. (1989). Stem cell theory of carcinogenesis. Toxicol. Lett. 49:283-295.
19. Nakano, S. and Ts'o, P.O.P. (1981). Cellular differentiation and neoplasia: characterization of subpopulation of cells with neoplasia-related growth properties of Syrian Hamster embryo cell culture. Proc. Natl. Acad. Sci. USA 78:4995-4999.
20. Potten, C.S. and Loeffler, M. (1990). Stem cells: Attributes, cycles, spirals, spitfalls and uncertainties. Lessons for and from the crypt. Devel. 110:1001-1020.
21. Trosko, J.E. (1994). Epigenetic biomarkers: Potentials and limitations. In: Biomarkers for Workers Health Monitoring. Joseph Henry Press, Washington, D.C., In Press.
22. Cha, R.S., Thilly, W.G., and Zarbl, H. (1994). N-nitroso-N-methylurea-induced rat mammary tumors arise from cells with preexisting oncogenic HrasI gene mutations. Proc. Natl. Acad. Sci. USA 91:3749-3753.
23. Brookes, P. (1989). Chemical carcinogens and ras gene activation. Molec. Carcinogen. 2:305-307.
24. Masui, T., Mann, A.M., Macatee, T.L., Okamura, T., Garland, E.M., Smith, R.A. and Cohen, S.M. (1992).

Absence of ras oncogene activation in rat urinary bladder carcinomas induced by N-methyl-N-nitrosourea or N-butyl-N-(4-hydroxybutyl) nitrosamine. Carcinogenesis 13:2281-2285.

25. Mass, M.J. and Austin, S.J. (1989). Absence of mutations in codon Gl of the Ha-ras oncogene in epithelial cells transformed in vitro by 7,12-dimethylbenz(a)-anthracene. Biochem. Biophys. Res. Commun. 165:1319-1323.
26. Aonuma, S., Ushijima, T., Nakayasu, M., Shima, H., Sugimura, T., and Nagao, M. (1991). Mutation induction by okadaic acid, a protein phosphatase inhibitor, in CHL cells, but not in S. typhimurium. Mutat. Res. 250:375-381.
27. Potter, V.R. (1978). Phenotypic diversity in experimental hepatomas: The concept of partially blocked ontogeny. Br. J. Cancer. 38:1-23.
28. Yuspa, S.H., and Morgan, D.L. (1981). Mouse skin cells resistant to terminal differentiation associated with initiation of carcinogenesis. Nature 293:72-74.
29. Potter, V.R. (1981). A new protocol and its rationale for the study of initiation and promotion of carcinogenesis in rat liver. Carcinogenesis 2:3175-3179.
30. Trosko, J.E., Chang, C.C. and Medcalf, A. (1983). Mechanisms of tumor promotion. Potential role of intercellular communication. Cancer Invest. 1:511-526.
31. Trosko, J.E., Chang, C.C., Madhukar, B.V. and Dupont, E. (1993). Oncogenes, tumor suppressor genes, and intercellular communication in the oncogeny as partially blocked ontogeny hypothesis. In: New Frontiers in Cancer

Causation. Iversen, O.H. (ed.), Taylor and Francis, Washington, D.C., pp. 181-197.

32. Bursch, F., Oberhammer, F., Schulte-Hermann, R. (1992). Cell death by apoptosis and its protective role against disease. Trends Pharmacol. Sci. 13:245-251.
33. Trosko, J.E. and Goodman, J.I. (1995). Intercellular communication may facilitate apoptosis: Implications for tumor promotion. Molec. Carcinogen. 11:8-12.
34. Goldsworthy, T., Campbell, H., and Pitot, H.C. (1984). The natural history and dose response characteristics of enzyme-altered foci in rat liver following phenobarbital and dimethylnitrosamine administration. Carcinogenesis 5:67-71.
35. Verma, A.K. and Boutwell, R.K. (1986). Effects of dose and duration of treatment with the tumor promoting agent, TPA, on mouse skin carcinogenesis. Carcinogenesis 1:271 276.
36. Pereira, M.A., Herren-Freund, S.L., and Long, R.E. (1986). Dose-response relationship of phenobarbital promotion of dimethylnitrosamine initiated tumors in rat liver. Cancer Lett. 32:305-311.
37. Maekawa, A., Onodera, H., Ogasawara, H., Matsushima, Y., Mitsumori, K. and Hayashi, Y. (1992). Threshold dose dependence in phenobarbital promotion of rat hepato carcinogenesis initiated by dimethylnitrosamine. Carcinogenesis 13:501-503.
38. Deml, E. and Oesterle, D. (1987). Dose response of promotion by polychlorinated biphenyls and chloroform in

rat liver foci bioassay. Arch. Toxicol. 60:209-211.

39. Trosko, J.E. and Chang, C.C. (1988). Nongenotoxic mechanisms in carcinogenesis: Role of inhibited intercellular communication. In: Banbury Report 31: Carcinogen Risk Assessment: New Directions in the Qualitative and Quantitative Aspects. Hart, R.W. and Hoerger, F.G. (eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., p. 139-174.
40. de Kok, T.M.C.M., Vaarwerk, F.T., Zwingman, I., Van Maanen, J.M.S. and Kleinjans, J.C.S. (1994). Peroxidation of linoleic, arachidonic and oleic acid in relation to the induction of oxidative DNA damage and cytogenetic effects. Carcinogenesis 15:1399 1404.
41. Aylsworth, C.F., Jone, C., Trosko, J.E., Meites, J. and Welsch, C.W. (1984). Promotion of 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis by high dietary fat in the rat: Possible role of intercellular communication. J. Natl. Cancer Instit. 72:637 645.
42. Chang, C.C., Jone, C., Trosko, J.E., Peterson, A.R., and Sevanian, A. (1988). Effect of cholesterol epoxides on the inhibition of intercellular communication and on mutation induction in Chinese hamster V79 cells. Mutat. Res. 206:471-478.
43. Hageman, G.J., Verhagen, H., Kleinjans, J.C.S. (1988). Butylated hydroxyanisole, butylated hydroxytoluene and tert,-butyldroquinone are not mutagenic in the Salmonella/microsome assay using new tester strains.

Mutat. Res. 208:207-211.

44. Witschi, H.R. and Morse, C.C. (1983). Enhancement of lung tumor formation in mice by dietary butylated hydroxytoluene: Dose-time relationships and cell kinetics. J. Nat. Cancer Instit. 71:859-866.
45. McGregor, J.F. (1982). Enhancement of skin tumorigenesis by cigarette smoke condensate following B-irradiation in rats. J. Natl. Cancer Instit. 68:605-611.
46. Saeter, G., Schwarze, P.E., Nesland, J.M. and Seglen, P.O. (1988). 2 Acetylaminofluorene promotion of liver carcinogenesis by a non-cytotoxic mechanism. Carcinogenesis 9:581-587.

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1. While there may be a statistical correlation between carcinogenicity and acute toxicity, there is considerable variation between compounds and species. In those cases where specific tissue injury is involved in acute toxicity it is not too surprizing that such a correlation exists. In any event, considerable caution is required in applying any response in a short term assay to chronic effects. In the dose-response relationship of carcinogenesis, the time of exposure may be more important than dose - this is expressed in the Druckery equation that indicates that the dose times a power of time (commonly a fourth or fifth power) is a constant. Another time related problem is that

there may only be a brief response to exposure. In rats, phenobarbital induces a short term burst of intense DNA synthesis in the liver only during the first week of exposure (Kolaja et al, in press). For phenobarbital and possibly other chemicals, the highest negative dose in the Kitchin-Brown paper is above what could be tolerated (MTD) on a daily basis.

2. The strand break assay based on alkaline elution is not a comprehensive analysis of DNA damage and was not correlated with other possible measures DNA damage. It does not provide conclusive evidence of the presence, or lack of DNA damage. However, there are other data which tend to provide directional support for the K-B findings. Detailed studies on DNA damage by non-genotoxic carcinogens such as phenobarbital have failed to show increases in DNA adducts by P32 labelling and other techniques (M. McClain, personal communication). Many of these compounds may be acting as promoters rather than as complete carcinogens. Strand breakage does provide a useful endpoint for generalized DNA changes which should be confirmed by other techniques.
3. The fact that there may be an apparent reduction of damage at low levels implies that there is a measurable background rate of damage. This is of considerable interest, but by giving results nomalized to the control values, the K-B paper does not address the background rate per se. We and others have found that the background DNA synthesis rates in rodent livers can be reduced significantly by dietary manipulations (Stevenson et al, in

press). The NCTR have found that dietary restriction may also have such an effect. The authors do not state whether food was withheld prior to gavage. Any difference in the way control and dosed animals were treated could introduce a systematic error which might be observable only at the lower doses. Jim Swenberg discussed at the 1994 summer meeting of the Toxicology Forum, the significance of specific DNA adducts which may be present arising from exogenous or endogenous sources of ethylene and ethylene oxide. He found that the endogenous sources can account for the adducts present at low exposure levels. Thus, two sources of DNA damage must now be considered (1) background or endogenous, which may be external exposure independant (2) exogenous -exposure dependent. Both these sources will modulate the dose-response relationship to varying degrees. Low dose linearity is unlikely to be present over a complete dose-range when two sources of damage exist..

4. The dose levels of toxicants are interesting. Typically in a bioassay the ratio of between the LD50 and daily dietary intake is not as great as the 1/1000 -1/50,000 used in the K-B study, i.e., they were exploring dose rates which fall below most bioassays. The authors state, correctly, that it is not possible to measure small changes in response in the usual design of bioassays. Figure 9 of the K-B paper shows the relationship of strand breakage to the LD50. It would be useful if this was also related to the responses in the bioassays to show where the lowest bioassay dose lies in relation to the LD50. For instance, say a compound has

an LD50 of 100mg/kg and is fed at 100 and 50 ppm (corresponding to about 5 and 2.5mg/kg/day). Then, the percentage of the LD50 would be 5 and 2.5% or on the linear part of figure 9. Few bioassays go below 1ppm (approximately 0.05mg/kg/day) or 0.05% of this hypothetical LD50 case, which is in the lower range of doses where the inflection occurs in figure 9. This raises two issues (1) while responses may be linear in the observed range, there is a possibility of an inflection occurring just below the lowest dose investigated. (2) The K-B data do not support the concept of linearity at very low doses. The data is also consistant with the predictions of the Sielken-Stevenson 'Invaders-Defender' discussion (see BELLE vol. 3 No. 2).

5. Many compounds have a dual action in the liver. Enzyme inducers also increase the level of synthesis of ascorbic and glucaric acids in rats, both of which are protective against liver damage, including DNA synthesis. In fact, the urinary excretion of these compounds has been used as an indirect marker of enzyme induction in rats (glucaric acid has been used in humans). There may be several other defense mechanisms which are activated prior to the onset of toxic changes, so a U-shaped response is not be surprising.
6. It should be possible to follow up the observations in this paper with additional experimentation using other biomarkers and differing exposure durations.
7. I am, of course, particularly interested in this paper because it fulfills the predictions of the 'invaders-defenders' concept,

which implies that if there is any interaction with molecules other than DNA, or if interaction occurs with non-functional DNA then at some point there may be an inflection or hockey stick in the dose-response curve. There are several defenses which may lead to a U-shaped response. For instance in the discussion of 'invaders-defenders' in (Belle Vol.3, No.2) we show that cell death can provide such a defense.

8. I feel that this paper raises a fundamental issue that requires vigorous follow-up, in relation to the linearity or otherwise of dose-responses. The economic importance to the USA of a low-dose non-linearity is substantial and should be given high priority for further study and development. The concept of low-dose linearity is not consistant with the multifactorial nature of the carcinogenic dose response. There are many defense mechanisms which may be activated at a higher rate than the formation of DNA adducts or mutations, particularly at low doses and thus lead to the presence of a U shaped response.

REFERENCES

1. K.L Kolaja. D.E.Stevenson, J.T.Johnson, E.F.Walborg,Jr. and J.E.Klaunig. Hepatic Effects of dieldrin and phenobarbital in male B6C3F1 mice and Fisher 344 rats: Species selective induction of DNA synthesis. Accepted for publication in the Proceedings of the Seventh International Conference on Carcingenesis and Risk Assessment, December 1-4, 1993 (Wiley-Liss).
2. D.E. Stevenson, J.P.Kehrer, K.L.Kolaja, E.F.Walborg,Jr.

and J.E. Klaunig. Effect of dietary antioxidants on dieldrin-induced hepatotoxicity in mice. Accepted for publication in Toxicology Letters.

Response by Kitchen and Brown

HISTORY OF THE STUDY

This dose-response study of 49 rodent carcinogens followed a 111 chemical study (including 62 rodent noncarcinogens) on cancer prediction published in Mutation Research in 1992 (1). Four biochemical assays (hepatic DNA damage as measured by alkaline elution, hepatic ornithine decarboxylase activity, hepatic cytochrome P-450 content and serum alanine aminotransferase activity) were discovered to be predictive of rodent carcinogenicity (a concordance of 73% versus only 53% concordance for the Ames mutagenicity test). In a prior study in isolated hepatocytes, Sina et al. (2) also obtained good DNA damage results with 91 chemicals (sensitivity 92% and specificity 85%). Because hepatic DNA damage after in vivo exposure was found to have a 100% specificity (all 62 noncarcinogens tested negative) and 100% positive predictivity (all 12 DNA damaging chemicals were rodent carcinogens) (1), a dose-response study of DNA damage was performed.

Our laboratory had also just finished a dose-response regression analysis on 22 different studies of 11 different promoters of carcinogenesis (3). This review article focused on experimental thresholds, concave dose-response curves versus unlogged dose and biologically based cancer risk assessment. Therefore, it was only natural to similarly analyze the 12 DNA damaging chemicals which we presumed to be initiators and not promoters of carcinogenesis.

Purpose and design of the study

The purpose of our study was to 1) determine the highest no observable adverse effect level (NOAEL) for all 49 rodent carcinogens, 2) determine the lowest observable adverse effect level (LOAEL) for the 12 DNA damaging carcinogens and 3) determine the dose-response relationship for the DNA damaging chemicals. It was not our purpose to prove or disprove "hormesis" or "U shaped dose-response curves". If this had been our purpose, the study would have been designed and performed much differently.

The DNA damage assay (alkaline elution)

The DNA damage assay used in this study is a biophysical technique which determines to what extent single stranded DNA passes through a polycarbonate filter containing 2.0 u pores during a 14 hour elution time with a pH 12.10 eluting solution. The DNA damage assay measures two classes of events 1) alkali labile sites (e. g. alkylation of phosphate groups of the DNA chain) and 2) existing single strand breaks (e. g. damaged bases removed by repair enzymes, depurination, depyrimidination, etc.). The sensitivity limit for DNA damage as measured by alkaline elution is about 0.25 Gy (about 3.0% of the LD50 for ionizing radiation). In the study discussed here our results showed significant DNA damage at 0.2%, 0.2% and 1.7% of the LD50 for 1,2-dimethylhydrazine, N nitrosopiperidine and 1,2-dibromoethane, respectively.

Positive aspects of the study

Despite the obvious importance of dose-response studies to the four part modern risk assessment paradigm (hazard identification, dose-response assessment, exposure assessment and risk characterization), it is both surprising and disappointing that so few dose-response animal studies of robust size are performed. Six major positive aspects of our dose-response study (Toxicology 88: 31-49 1994) are 1) the better interpretability that comes from using a biological endpoint (hepatic DNA damage) with 100% positive predictivity and specificity, 2) the 6 orders of magnitude over which experimental data is available (Figure 1), 3) the 5 orders of magnitude in which the study chemicals damaged hepatic DNA, 4) the 8 orders of magnitude over which the data has been analyzed and extrapolated 5) the NOAEL for 49 different rodent carcinogens and 6) the LOAEL for 12 rodent carcinogens.

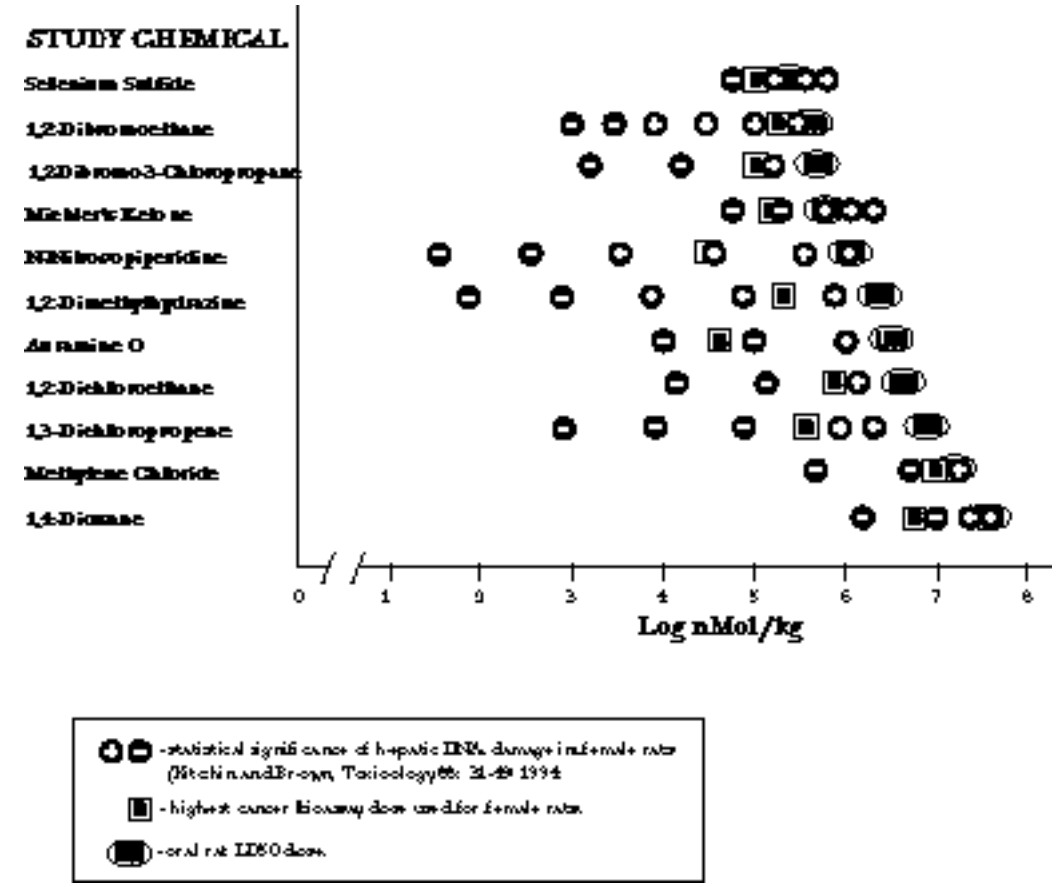

Figure 1. Logarithmic plot (in nmol.kg) comparing the dosages causing DNA damage, cancer and 50% acute lethality.

Generalized responses to the commentaries

Possible stimulation of DNA repair systems at low doses

Both Drs. Hart and Turturro, and Dr. Green's group at USFDA hypothesize that stimulation of DNA repair systems occurred by low doses of several of the study chemicals. Eight experimental values are numerically but not statistically significantly below the control values. At any sampling time the existing amount of rat liver DNA damage will be a dynamic balance between adverse DNA damaging events and the opposing beneficial DNA repairing systems (as pointed out by Drs. Stevenson, Hart and Green et al.). While this "stimulation of DNA repair" hypothesis is a plausible and elegant interpretation of the experimental measurements found to be below control values, we do

not favor this particular hypothesis ourselves. The eight compounds showing experimental values lower than control values (1,2-dibromo-3-chloropropane, 1,2-dichloroethane, 1,4-dioxane, methylene chloride, auramine O, selenium sulfide, butylated hydroxytoluene, and N nitrosopiperidine) were first selected as rodent carcinogens, and second demonstrated to cause DNA damage at mid or high doses. For the "stimulation of DNA repair" interpretation to be correct, the eight compounds must also 1) actually stimulate DNA repair systems at low doses (no data for this exists at present) and 2) have the putative stimulation of DNA repair systems quantitatively larger than the already demonstrated DNA damaging potency of these chemicals. Believing both items 1 and 2 are true without any experimental evidence calls for a considerable leap of faith. Our preferred interpretation of the animal experimental values which were below control values is that they are simply random variation around the control values. None of the experimental values numerically lower than control values are statistically significant either individually or as a group as shown by Dr. Svendsgaard in his independent statistical analysis of our data. By a Fischer's exact test, 8 experimental values found to be below control values per 12 total measurements are not statistically significant. The data set would have to be 11 or more experimental values below control values per 12 measurements to reach a $P < 0.05$ level of significance.

Logarithms

Many scientists who were first educated in the disciplines of pharmacology, toxicology, endocrinology or biochemistry favor the use of logged dose or concentration in their studies. Other scientists from fields such as radiation biology, statistics, mathematics, and risk assessment often favor unlogged dose or concentration. In the present study the coefficient of the linear term of the quadratic regression equation is positive for all 12 chemicals on unlogged dose, but is negative for all 12 chemicals on logged dose. Much of BELLE's interest in our dose-response study seems to be centered on Figure 14's dose-response curve of an "average DNA damaging carcinogen". Therefore, it is important to state again that in our extrapolation of regression equations the negative deviations below control values happen only with logged dose and not with unlogged dose. Our paper also presented the unlogged quadratic regression equation of an "average DNA damaging carcinogen" as:

$$Y = 1380 \times (\text{dose in nmol/kg}) - 968 \times (\text{dose in nmol/kg})2$$

The r2 value is 0.947 and the P-value of the quadratic term is 0.185. On a linear plot, this regression line is perfectly linear from the zero dose point up to about 105 nmol/kg (the first 5 orders of magnitude of unlogged dose), and then

the regression line begins to curve downward in a convex fashion at about 106 nmol/kg.

Law of Mass Action

This well known law is a widely used cornerstone of chemistry, biochemistry, pharmacology, toxicology and endocrinology. Good elementary treatments of its use in the biological sciences can often be found in textbooks such as Casarett and Doull's Toxicology, Goldstein, Aronow and Kalman's Principles of Drug Action, and Goodman and Gilman's The Pharmacological Basis of Therapeutics. The law of mass action predicts that when plotted versus either unlogged and logged dose, a dose-response curve will initially slope upward (concave) when one starts from the lowest of doses. Interestingly, the results of our regression on unlogged dose presented in Table 2 showed many negative quadratic coefficients, and thus downward slope (convex) shaped dose-response curves. This is the exactly the opposite of predictions based on the law of mass action. The dose response curve on logged dose (Figure 14) did eventually slope upward (concave), as predicted by the law of mass action.

Individual Responses To The Commentaries

Dr. Edward Calabrese

Environmental policy is often driven by cancer risk assessment which in turn is driven by the assumptions that most or all rodent carcinogens act in a genotoxic manner and that linear low dose extrapolation is appropriate for all rodent carcinogens. Therefore, it does seem to be a reasonable approach to use genotoxic endpoints of high positive predictivity to determine the dose-response relationship of rodent carcinogens in the low dose region. Rodent hepatic DNA damage data could be used for such risk assessment purposes. Such experimental information would be germane to the genotoxic process and stages of carcinogenesis but not, of course, to the nongenotoxic processes and stages of carcinogenesis.

Dr. David Svendsgaard

No Y-intercept was used for these regressions because the (x = zero dose, y = zero additional DNA damage caused by the zero dose) data point is the only (x,y) ordered pair we know with certainty. All other ordered pairs used for regression analysis contain experimental variation and error. The confidence limits merging at the 0 nmol/kg dose point of Figure 14 are a consequence of the statistical package used (SAS System Release 6.06) and not by the authors' preference. The between chemical variation for the linear and quadratic regression coefficients was fairly high (Table 2 of original study) for unlogged dose and considerably less for logged dose. To state this in biological

terms the DNA damaging potency of the study chemicals ranged from a low of 0.45 mg/kg for 1,2 dimethylhydrazine to a high of 2550 mg/kg for 1,4-dioxane. On a percent LD50 basis the DNA damaging potency ranged from a low of 0.2% of the LD50 for both N-nitrosopiperidine and 1,2 dimethylhydrazine to a high of 60% of the LD50 for 1,4-dioxane, methylene chloride, Michler's ketone and selenium sulfide.

Dr. Rick Cothern
The total number of animals used in the study was 1099, of which 426 received treatment chemicals and 673 were controls. The number of dose levels per chemical available for regression analysis ranged from 4 to 7 with a mean of 5.3.

Dr. James Trosko
Some of the complexities of multiple causes and stages of carcinogenesis are presented in this commentary which centers on the mechanistic controversy between the genotoxic and the nongenotoxic schools of thought. In our article on DNA damaging rodent carcinogens we found with unlogged dose 9 out of 11 chemicals had a negative quadratic coefficient (and thus were convex curves). However in our prior regression study of unlogged dose of 11 chemicals which were promoters of carcinogenesis, we found that there was statistically significant evidence for a positive quadratic coefficient (concavity of dose-response curves) for 6 chemicals; 4 chemicals did not show significant positive quadratic coefficients (3). Thus for unlogged dose, these two studies indicate that DNA damaging chemicals (genotoxins, initiators of carcinogenesis) generally had convex dose-response curves which sloped downward with increasing dose; promoters of carcinogenesis generally had concave dose-response curves which sloped upward with increasing dose.

Dr. Ron Hart
Carbon tetrachloride increased both hepatic ornithine decarboxylase activity and serum alanine aminotransferase activity and thus was classified as a cell toxicity or cell necrosis type of carcinogenic agent in our 1992 cancer prediction study (1). Aflatoxin B1 was quite active inducing hepatic cytochrome P-450, hepatic ornithine decarboxylase activity and serum alanine aminotransferase activity. Aflatoxin B1 numerically increased rat hepatic DNA damage by 79% and 39% at 1 and 3 mg/kg, respectively. By statistical tests of significance, 1 mg/kg of aflatoxin was not significant by a paired t-test but was significant by an ANOVA (P=0.033), neither statistical analysis showed significance of aflatoxin B1 at a dose of 3 mg/kg. These results are the basis for not classifying aflatoxin B1

as positive for rat hepatic DNA damage.

Dr. Donald Stevenson
The DNA damage assay used provides information about a number of types of genotoxicity (e. g. phosphotriesters, single strand breaks, depurination, depyrimidination and damaged bases removed by repair enzymes). One advantage of the alkaline elution technique is that it can detect genetic events at any position along the length of DNA strands, unlike many other point mutational assays. Food was not withheld from the experimental animals either before or after chemical treatment.

SUMMARY

We wish to suggest possible future uses of this type of experimental data:

1) Experimental data of high positive predictivity could be used in cancer risk assessment to shed light on the extrapolation to low environmental doses issue (Drs. Kitchin and Calabrese). Data from the three most potent chemicals of our dose-response study (1,2-dimethylhydrazine, N-nitrosopiperidine and 1,2-dibromoethane) are available to use in cancer risk assessment. Second choice chemicals might include auramine O, 1,2-dichloroethane and 1,3-dichloro-propene.

2) Genotoxic data should be used to model the dose-response relationship for the genotoxic stages of multistage carcinogenesis (e. g. initiation and possibly progression). Nongenotoxic data should be used to model the nongenotoxic stages of carcinogenesis (e. g. promotion of carcinogenesis, regenerative hyperplasia induced initiation, cell proliferation induced initiation).

3) Genotoxic data, such as our 1994 study, may provide an additional experimental basis for selection of cancer risk assessment models and extrapolation from animal cancer data to human cancer risk.

REFERENCES

1. Kitchin, K.T. Brown, J.L. and Kulkarni, A.P. Predictive assay for rodent carcinogenicity using in vivo biochemical parameters: operational characteristics and

complementarity. Mutation Research 266: 253-272 1992

2. Sina, J.F., Bean, C.L., Dysart, G.R., Taylor, V.I. and Bradley, M.O. Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential. Mutation Research 113: 357-391 1983
3. Kitchin, K.T., Brown J.L. and Setzer, R.W. Dose-response relationship in multi-stage carcinogenesis: Promoters. Environmental Health Perspectives 102 Suppl 1: 255-264 1994

Final Thoughts on the Debate

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Debate of the Kitchin & Brown paper and the authors' interpretation of their data was considered important since the database offered a broad consideration of the chemicals tested, an exceptionally wide range of doses (up to six orders of magnitude of dose), a potentially important endpoint (DNA damage), and the fact that the response for logged dose indicated consistently less damage in the low dose range for most of the agents tested than in the control group.

While Kitchin & Brown did not support the hypothesis that a low dose of genotoxic agent might produce beneficial responses (i.e. less damage), it was felt that their paper and interpretation, needed further discussion.

What was learned from the debate? The present data do not have the statistical power to resolve the question of whether the "protective" response at low doses is real or simply due to chance. While this dip below the control was a generally consistent response a study specifically designed to assess this question would need to be conducted. Without such a follow-up study little progress can be made.

I was also impressed with the sentiment offered by Hart and Turturro that it is "more fruitful to explain results rather

than explain them away". To this end, the commentaries of Green, Trosko, Hart, and Stevenson provide different but potentially complementary toxicological, mechanism based hypotheses that offer follow-up investigators specific ideas for evaluating the reported dose response relationships. Such independently offered commentaries, which provide a glimpse into the independent creative process whereby plausible hypotheses are generated, require follow-up if the original findings are to be confirmed and extended.

Finally, while the implications of such experiments are obviously important for those working in the area of cancer mechanism and risk assessment, a re-reading of the Trosko commentary will emphasize that regardless of the eventual shape of the dose-response curve, acceptance of any paradigm is a risky proposition given the complexity of carcinogenic processes.