Thresholds of Carcinogenicity in the ED01 Study

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The results of the articles on the carcinogenicity of 2-acetylaminofluorene (J. H. Farmer et al., 1980, J. Environ. Pathol. Toxicol. 3, 55–68; N. A. Littlefield et al., 1980, J. Environ. Pathol. Toxicol. 3, 17–34) in approximately 25,000 female mice were reanalyzed by the procedure proposed earlier (W. J. Waddell, 2002, Toxicol. Sci. 68, 275–279) using the Rozman scale (K. K. Rozman et al., 1996, Drug Metab. Rev. 28, 29–52). In contrast to some conclusions of the lack of a threshold for carcinogenesis that have been made in the past from this study, this reanalysis showed a clear and consistent threshold for bladder neoplasms at about 10^10 molecules/kg/day and for liver neoplasms at about 10^11 molecules/kg/day. The slopes of the dose-response curves for bladder neoplasms from 17 months through 33 months were consistently very steep, while those for liver neoplasms increased from a shallow slope at 18 months to a steep slope at 33 months. This is interpreted to indicate that the mechanism of carcinogenesis may be different in the two organs. A linear response for percentage tumors plotted against dose on a logarithmic scale is confirmed by this analysis, which is based on the fundamental principle that chemical potential effects a linear response. Furthermore, this application continues to show a sharp threshold for carcinogenesis. The implications of these observations should be important in the extrapolation of results from animal experiments to human risk assessment.

Key Words: thresholds; carcinogenicity; 2-acetylaminofluorene; 2-AAF; ED01; bladder neoplasms; liver neoplasms.

Perhaps the largest published study in experimental animals for carcinogenicity was that of 2-acetylaminofluorene, frequently referred to as the “ED01 Study” (Farmer et al., 1980; Littlefield et al., 1980). In this study of approximately 25,000 female, BALB/c StCrIf C3Hf/Nctr mice, there were significant tumors of the liver and bladder. There appeared to be a threshold for bladder tumors, but not for liver tumors when plotted on a linear scale for dose (see Klaassen, 2001, or Hayes, 2001, for summaries and discussion). The overall conclusion of the study was originally summarized by Gaylor (1980, p. 179) as: “The incidence of bladder tumors dropped off sharply as the dosage of 2-AAF was reduced. However, liver tumors showed a nearly linear response over the experimental dose range, thereby dissolving [sic] any notion of a threshold dose.” These results have been widely debated by many individuals and groups as to whether chemical carcinogens may or may not have a threshold. In the present report, the results of the ED01 study were reanalyzed by the procedure proposed by Waddell (2002) using the Rozman scale (Rozman et al., 1996). The Rozman scale was a brilliant concept and an enormous advancement for toxicology; however, no attempt was made to extrapolate beyond the data points that were available. The procedure proposed by Waddell (2002) is based on fundamental laws of chemistry, namely that the chemical potential of a substance effects a linear response. Consequently, percent tumor response can be plotted linearly against logarithmic dose, from 0–100% tumors, to provide a threshold dose at zero tumors. The results confirm the procedure and indicate a clear threshold for carcinogenicity in both sites in the ED01 study, but the mechanism may be different.

MATERIALS AND METHODS

Data from Tables 3 and 4 of the Littlefield et al. (1980) publication were used for the calculations on neoplasms in the bladder and liver, respectively, of mice sacrificed at each of the different time intervals. Data from Tables 1 and 2 of the Farmer et al. (1980) publication were used for the calculations on neoplasms of the bladder and liver, respectively, in dead, moribund, and sacrificed mice at each of the different time intervals. The doses of 2-acetylaminofluorene in the feed in ppm were converted to molecules/kg/day by using its molecular weight, and assuming the weight of adult female Balb/c mice as 32 g with consumption of 5 g of feed daily (Dr. William Allaben, personal communication); i.e., 0.156 × ppm = mg/kg/day. The percentage of mice with tumors in the control mice was subtracted from the percentage at each of the dosage levels. These data were then plotted on the abscissa with the Rozman scale (Rozman et al., 1996) and linearly on the ordinate for percentage tumors as proposed by Waddell (2002) using SlideWrite software (Advanced Graphics Corporation, Inc. Encinitas, CA). The slopes, intercepts with the abscissa and correlation coefficients were calculated by the SlideWrite software. A linear fit was chosen for each dose response based on the results of Waddell (2002), and Waddell (in press); this also was found to be the best fit for the results in this article.

RESULTS

Table 1 shows the results of the calculations described in the Methods section for molecules/kg/day and the percent tumors from the publications of Farmer et al. (1980) and Littlefield et al. (1980) that were used in the figures.
Figures 1–4 show the results of the calculations for bladder tumors in mice sacrificed at 17, 18, 24, and 33 months. The percentages of tumors at doses that were very close to those in the controls (10^10.21 to 10^11.2% above control values, as shown on the graphs) were not used for determination of the dose-response curve. At 17, 18, and 24 months there were only two data points for determination of the dose-response curve; at 33 months there were four points. The slopes of these lines were similar to those in the sacrificed animals; they were also very steep, ranging from 249–272% per 10^1 molecules/kg/day. The correlation coefficient for a linear fit for the line with four points was 0.983; for the line with three points it was 0.974. Their intercepts with the abscissa were from 10^19.4 to 10^19.6 molecules/kg/day, which are identical to those found for the sacrificed animals alone.

Figures 5–8 show the bladder neoplasms for all (dead, moribund, and sacrificed) mice at 17, 18, 24, and 33 months. The percentages of tumors at doses that were very close to those in the controls (10^1.1% to 10^1.5% above control values, as shown on the graphs) were not used for the determination of the dose-response curve. At 17 and 18 months there were only two data points for determination of the dose-response curve; at 24 months there were three points; at 33 months there were four points. The slopes of these lines were similar to those in the sacrificed animals; they were also very steep, ranging from 249–272% per 10^1 molecules/kg/day. The correlation coefficient for a linear fit for the line with three points was 0.974; for the line with four points it was 0.983. Their intercepts with the abscissa were from 10^19.4 to 10^19.6 molecules/kg/day, which are identical to those found for the sacrificed animals alone.

Figure 9 shows the liver neoplasms in the sacrificed mice at 18, 24, and 33 months. The results at 17 months were not used because there was no significant increase in tumors above the control at any of the doses at this time interval. In the 18-month sacrifice interval, the tumors at 30 and 35 ppm were not used.

### Table 1: Data Used for Calculations and Plotted in Figures

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<thead>
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<th>Dosages ppm in feed</th>
<th>mg/kg/day</th>
<th>molecules/kg/day</th>
<th>Fig. 1</th>
<th>Fig. 2</th>
<th>Fig. 3</th>
<th>Fig. 4</th>
<th>Fig. 5</th>
<th>Fig. 6</th>
<th>Fig. 7</th>
<th>Fig. 8</th>
<th>18 Mo</th>
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<td>c</td>
<td>10.3</td>
<td>37.5</td>
<td>65</td>
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a Percentages for animals receiving 2-acetylaminofluorene are minus percentages at zero dose.
b Datum not plotted. See Results for explanation.
c No animals sacrificed.
for the curve statistics because they were less than 1% above the control values. In contrast to the slopes for the bladder curves, these slopes increased with time from 8% per 10^1 molecules/kg/day at 18 months to 68.7% per 10^1 molecules/kg/day at 33 months. The intercepts with the abscissa ranged from 10^{19.0} molecules/kg/day to 10^{19.1} molecules/kg/day. The correlation coefficients for a linear fit ranged from 0.912 to 0.978; these fits were based on five points in the 18-month sacrifices, seven points in the 24-month sacrifices, and six points in the 33-month sacrifices.

Figure 10 shows the liver neoplasms in all (dead, moribund, and sacrificed) mice at 18, 24, and 33 months. The results at 17 months were not used because there was no significant increase in tumors above the control at any of the doses at this time interval. In the 18-month sacrifice interval, the tumors at 30 and 35 ppm were not used for the curve statistics because they were less than 1% above the control values. There was an obviously spurious datum, visually, at 35 ppm of 55% tumors at 33 months that was omitted from the curve fit statistics. Inclusion of this point slightly decreased the slope (80.7% to 68.7%) and intercept (10^{19.0} to 10^{19.1} molecules/kg/day), but considerably decreased the correlation coefficient (r = 0.962 to 0.647). An advantage of this procedure is that visual inspection, plus curve fit statistics, allow the identification of obviously spurious data. In agreement with the slopes for the sacrificed mice, the slopes increased from 18.3% per 10^1 molecules/kg/day in the 18-month animals to 80.7% per 10^1 molecules/kg/day in the 33-month animals. The intercepts with the abscissa ranged from 10^{19.0} to 10^{19.2} molecules/kg/day, which are very close to those for only the sacrificed animals. The correlation coefficients for a linear fit ranged from 0.962 to 0.967; these fits were based on five points in the 18-month mice, seven points in the 24-month animals, and six points in the 33-month mice.
DISCUSSION

These calculations are in agreement with the previous publications showing clear thresholds for the carcinogenicity of flavors (Waddell, 2002) and N-nitrosodiethylamine (Waddell, in press). This agreement in a study, such as the ED01, with a very large number of animals lends strong further support to the concepts proposed in those earlier studies.

The question has always been the shape of the dose-response curve; linear, logarithmic, and other scales have been used on the abscissa and ordinate in an effort to find a solution to the question. A logarithmic scale for the doses is the only one that is in agreement with chemical thermodynamics. The force or effect that a chemical exerts on a system is directly proportional to the chemical potential of that chemical, and the chemical potential is directly proportional to the logarithm of its concentration. $\mu = RT \ln (a)$, where $\mu$ is the chemical potential of substance x when its chemical potential in the aqueous standard state is zero, $R$ is the gas constant, $T$ is the absolute temperature, and $a$ is the activity of substance x. Concentration is directly proportional to activity. This was pointed out and discussed some years ago in reference to the controversy over whether it was more correct to average multiple pH values directly or to convert them to hydrogen ion concentration for averaging and then convert that number back to pH (Waddell and Bates, 1969). That argument applies just as well to any chemical in a system. It is critically important when there are multiple equilibria, which is of course the case in a living cell. When a chemical is introduced into a system with multiple components that are all in mutual equilibrium, that chemical may have a direct reaction with only one or a few of the components. However, there may well be secondary adjustments in other equilibria following the changes in concentration of the substrates and products of those direct reactants.
The best way to express the relative effects of these changes in concentration is to consider the changes in their chemical potentials. The change in chemical potential from a change in concentration is relative to the initial concentration; i.e., the linear change in the logarithm of the concentration.

The Rozman scale (Rozman et al., 1996) was an enormous scientific advancement for toxicology; it is most appropriate for the abscissa because it not only uses a logarithmic scale for doses, but also uses molecules instead of mg, ppm, etc., which allows comparisons between chemicals with different molecular weights. Furthermore, it has a scale for doses down to a single molecule \(10^{9}\), which allows placing all doses in perspective.

Agreement on the scale for expressing dosage still requires a determination of the scale for effect, i.e., the ordinate. The theoretical considerations outlined above indicate that the ordinate should be linear; that is, a linear effect should result from the logarithm of the concentration. The experimental results from the present series of articles agree with this thinking and, for now at least, establishes a linear scale for percentage tumors as correct for carcinogenicity in animal studies. For methyl eugenol the range of four doses from 2–68% tumors fit a linear function with a correlation coefficient of 0.999983 (Waddell, 2002). For N-nitrosodimethylamine there were 6 doses ranging from 5–80% tumors that fit a linear function with a correlation coefficient of 0.993 (Waddell, in press). In the present report there are several examples of linear fits for five, six, and seven points with a range as large as from 2–99% tumors with a correlation coefficient of 0.983 (Fig. 8). This is remarkable agreement for biological experiments. Assumption of any other scale now, it would seem, incurs the burden of proof.

The inclusion of studies with only two data points to establish a linear fit in this report can be justified for two reasons. First, the studies with more than two data points were all linear. Secondly, the intercepts with the abscissa of those with only two points agreed with those in the series that had more points.

Subtracting the percentage of tumors in the control group of animals is clearly justified because these tumors obviously had nothing to do with the tumors resulting from the carcinogen added to the diet. To include these tumors in the experimental results would simply confound the results for the experimental carcinogen. Furthermore, not including data with only about 1% increase above control in the curve fit statistics can be justified on the basis of experimental variability.

There are three important observations that are realized from this analysis. First, it appears that only two reliable data points are needed for the determination of the slope of the dose-response curve and its threshold dose. This results from the apparent confirmation from this and the previous articles (Waddell, 2002, in press) that the carcinogenic response (% tumors) is linear when dose is on a logarithmic scale. There are many studies with only two data points, e.g., the National Toxicology Program (NTP) Technical Reports (TR-) on over 509 substances, about half of which have two data points for percentage tumors. These NTP studies now can be analyzed usefully for determination of their thresholds and comparison with daily exposures similar to that done by Waddell (2002) for flavors. This analysis currently is in progress by the author.

Secondly, the thresholds could be determined amazingly closely at 17 or 18 months. These differences between either 17 to 33 months for bladder tumors \((10^{19} \text{ to } 10^{20} \text{ or } 2.51 \times 10^{19} \text{ to } 3.98 \times 10^{19} \text{ molecules/kg/day})\) and 18 to 33 months for liver tumors \((10^{19} \text{ to } 10^{20} \text{ or } 1 \times 10^{19} \text{ to } 1.58 \times 10^{19} \text{ molecules/kg/day})\) were less than two-fold. This suggests that at whatever time two reliable data points for tumors are available from an experimental study, the threshold can be calcuated.

Thirdly, the slopes of the bladder tumors were remarkably steep and did not change appreciably with time. The slopes for liver tumors clearly increased with duration of intake of the carcinogen. The only interpretation that this author can make of this, at this time, is that the mechanisms of carcinogenesis are different in the two sites. In the bladder cells, once the threshold is exceeded (and that system or equilibrium is overwhelmed) tumor development proceeds rapidly and perhaps unimpeded. In the liver cells, perhaps some modifying effect intercedes after the threshold is exceeded. The small decrease in the x intercept (threshold) with time of treatment could be due just to noise in the data. However, its constancy is intriguing and deserves some further thought and comment. For liver tumors there were decreases in the threshold at 24 and 33 months from that at 18 months. The intercept for bladder tumors in sacrificed mice was constant at 17, 18, and 24 months; then at 33 months it decreased to a value 0.63 times that at the earlier time intervals. In all the mice (dead, moribund, and sacrificed) with bladder tumors the intercepts were the same as in the sacrificed mice at 17, 18, and 33 months; at 24 months the value was intermediate. These latter values are shown graphically in Figure 11; the equation for the best fit for these data (calculated by the SlideWrite software) and its correlation coefficient are shown on the graph. The threshold for a mouse with one month of treatment would be about \(10^{20.6} \text{ molecules/kg/day}\). This range of about 40-fold \((1 \times 10^{19} \text{ to } 3.98 \times 10^{20} \text{ molecules/kg/day})\) is impressively small for a lifetime (one month versus 33 months) of exposure to the carcinogen for an animal. There are too few data here to attach much significance to this calculation. The slope could even be linear (indeed, that correlation coefficient was 0.993), which would define an even smaller change in the threshold from one month to 33 months of exposure. In any event, it appears that there is a relatively large absolute threshold below which no tumors would appear within a normal lifespan.

It should be recognized that this discussion applies to the conditions of this experiment. Extrapolation from these data to any hypothetical prediction of the number of tumors that should be produced in a population of 1 million people (or any number chosen) is unwarranted and exceeds the information.
available. These data do provide strong evidence that there is a threshold below which no tumors will be produced. Therefore, the animal experiments should be used to provide evidence for the safety of the chemicals if human exposure is below that threshold.

In summary, the present article shows clear thresholds for both bladder and liver in the ED01 study and lends further support to the previous analyses that have found clear thresholds for carcinogenicity for a variety of substances in animal experiments (Waddell, 2002, in press). Reanalysis of other studies is underway. The presence of a threshold for carcinogenesis from chemicals has extensive implications for human risk assessment.

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REFERENCES


