

The Effect of Different Tumor Groupings on Findings of Anticarcinogenic Responses in Long-Term Rodent Bioassays

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Many investigators have found that there is a decrease in tumor rates at some sites when rodents are exposed to some chemicals. The generality of this finding of anticarcinogenicity has been questioned. In this study, we evaluate the effect of several alternative ways of grouping the 3000 tumor types in the Cancer Bioassay Data System (CBDS) database of carcinogenesis test results into a limited number of classes on findings of anticarcinogenicity. We also study the influence of random variation of tumor rate on the apparent anticarcinogenic effects of specific chemicals. We compare the numbers of chemicals classified as anticarcinogenic in (1) our “standard” classification system, (2) a modification of that system to correct some deficiencies in the CBDS database pointed out by Dr. J. Haseman, (3) an alternative classification system developed by Dr. K. S. Crump and colleagues, (4) the number of animals displaying at least one tumor, or (5) the total number of tumors appearing in all animals in control and dosed groups. Although there is a difference in the number of chemicals classified as anticarcinogens by these alternative classification schemes, all of them show a statistically significant increase in the number of anticarcinogenic responses above the random rate predicted by a Monte Carlo simulation of the rodent bioassay. The number of anticarcinogenic responses is similar in our standard classification, our modified classification, and a classification scheme developed by Crump, though specific site/organs may differ. This arises because these schemes use approximately the same number of tumor groups (about 100). If the number of tumor groups decreases (for example, in the total tumors or tumor-bearing animal schemes), the number of anticarcinogenic responses decreases because of the decrease in overall sensitivity of the test. A scheme which combines tumor types can be useful by integrating carcinogenic and anticarcinogenic responses to exposure. At

the same time, combination may hide important biological information. We urge consideration of both increases and decreases when evaluating the likely human effects of exposure. © 2002 Elsevier Science (USA)

INTRODUCTION

Long-term treatment of rodents with high doses of chemicals clearly has led to decreases in tumor rates at specific sites (e.g., Weinberg and Storer, 1985; Davies and Monro, 1994; Haseman and Johnson, 1996; Dunnick *et al.*, 1996; Elwell *et al.*, 1996). For example, when rats were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (“dioxin”), there was an increase in liver tumors (at high doses) but a decrease in other tumors (Kociba *et al.*, 1978). When mice were exposed to 2-acetylaminofluorene (2-AAF) there was an increase in many tumors at high doses. But at low doses bladder tumors were not seen below a threshold (Littlefield *et al.*, 1979), and reticular cell sarcomas decrease (Pompei, 2001), although liver tumors still showed an increase. We have been studying whether a similar behavior can be seen in the bioassays of the National Toxicology Program (Linkov *et al.*, 1998a,b, 2000, Gray *et al.*, 2000). We have shown that the decreased tumor rates (anticarcinogenicity) are sufficiently common that they cannot be completely explained either by random variations in tumor rates that cause a few of the multiple comparisons undertaken in evaluating a bioassay to be spuriously anticarcinogenic or by compound-induced decreases in body weight or decreases in survival of treated animals. We concluded that the decreases in tumor rates must have biological significance. However, it has been suggested that the observed decreases may merely be an artifact of the manner in which tumor types and sites were combined into classes for statistical analysis. In this paper we explore this possibility further.

An issue of long standing, but little study, is how to assign of tumors to different classes for evaluation of rodent bioassays. For 13 years our group has used a

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consistent grouping of tumors into 102 classes (Bailar *et al.*, 1988). This particular grouping into classes has been criticized by Haseman (1998, 2000) because (*inter alia*) (i) chromophobe adenoma and pituitary adenoma were put into separate classes although they belong in a single class; (ii) some tumors that were distinct were put into the same class; (iii) a distinction between liver adenoma and carcinoma could lead to spurious anticarcinogenic responses as adenomas progress to carcinomas (Gray *et al.*, 2000); (iv) Linkov *et al.* (1998a,b) had included a number of studies which existed on the data base provided to us, but had never been approved for "hard copy" publication; and (v) the names of some of the tumors in the Cancer Bioassay Data System (CBDS) database were not understood by Dr. Haseman (or by us).

Criticisms (i) and (ii) were certainly valid. Nevertheless, combining unrelated tumors into a single class cannot lead to spurious effects although it can lead to a lack of sensitivity. Separating similar tumors into two classes cannot lead to spurious results if it is either done randomly or done consistently. But spurious results can, and in the case of pituitary tumors do, appear if the pathologist is informed which animals were dosed and which not. This unfortunate NTP practice prevents a "blind" evaluation. It seems that there was a pathologist bias: in some cases all the dosed animals were in one class, and all the control animals in another. For pituitary tumors this is obvious. For liver tumors it is still not completely certain whether pathologists distinguish adenomas and carcinomas sufficiently reliably to avoid the problem.

PURPOSE OF THIS PAPER

In Linkov *et al.* (1999) we corrected the results of Linkov *et al.* (1998a,b). We reduced (modified) the number of classes to 100 by combining all liver tumors in one class and all pituitary tumors in another. We can then evaluate the influence on judgment of anticarcinogenic responses of Dr. Haseman's suggestions. We have also obtained an alternative classification scheme constructed by Dr. K. Crump and colleagues in consultation with Dr. Haseman (Crump, 1999). This scheme seems to be based upon the NTP approach used in the TDMS database, with a few tumor classes left over (see Wilson, 2000). Since a common response in bioassays is an increase in tumor rates at some sites and a decrease at others, we also consider two classification approaches that integrate increases and decreases in tumor rates. First, we count all animals with *any* tumor. We call this scheme "tumor-bearing animals." Since we focus on anticarcinogenicity the fact that the animals used in standard cancer bioassays have relatively high background tumor rates and often more than one type of tumor makes this approach somewhat less sensi-

tive in detecting anticarcinogenic effects. We therefore evaluate a final classification where all tumors in an animal group are counted, even when there is more than one tumor in an animal. In this work we included all studies in the database (even those without "hard copy"), notwithstanding Dr. Haseman's criticism (iv) since data should not be eliminated without clear reason and the justification for exclusion of these studies has not been provided to us. But we showed in Linkov *et al.* (1999) and show in this paper that the fraction of chemicals that are found to be anticarcinogenic is almost the same whether or not we exclude these disputed studies.

Methods

We use the database that we have used previously in the publications of this group (e.g., Byrd *et al.*, 1990; Gray *et al.*, 1995; Linkov *et al.*, 1998a,b, 1999). This is the CBDS database of chemicals tested by the National Toxicology Program prior to 1983. From this we identified 312 chemicals tested by NCI/NTP prior to 1983 that satisfied the following criteria:

- At least one control group;
- Long-term experiments (>70 weeks);
- Food, water, and gavage routes; and
- Tested on both mice and rats.

In response to concerns of Haseman (2000) we have also run the analysis with a few more restrictive criteria. We exclude chemicals in the database for which NTP issued no report in "hard copy" and those chemicals for which there is a discrepancy between the database provided to us and the NTP hard copy report. The results of the more restrictive analysis are only slightly altered and are available at <http://phys4.harvard.edu/~wilson/tumorgrouping/extra.html> (Wilson, 2000). The only notable change is the removal of *tert*-butyl alcohol from the group of chemicals classified as anticarcinogenic by schemes 1, 2, and 3 (Table 2).

Tumor Classification

In this database there are over 3000 combinations of tumor type and tumor sites. In addition, the classification and nomenclature of tumors were not consistent across the years of experiments covered by the CBDS. This means that tumors must be combined into classes for analysis. The goal of classification is maintaining the biologic integrity of each class while avoiding problems like inconsistent pathologic diagnosis. We evaluated the following five classification schemes (complete descriptions in Wilson, 2000).

1. Our *standard* classification (due originally to Bailar *et al.*, 1988) of 102 groups described in several earlier papers (e.g., Byrd *et al.*, 1990; Gray *et al.*, 1995;

Linkov *et al.*, 1998a,b, 1999) and listed here in the appendix. Among these 102 categories (classes), 66 were classified as malignant primary neoplasms and 36 as benign primary neoplasms.

2. The *modified* classification is similar to the standard one but all pituitary tumors (previous classes 10 and 36) are combined into one class and all liver tumors (adenomas, carcinomas, and other neoplastic lesions) previously in our classes 7 and 64 are combined into one class. This “modified” classification scheme or combination scheme was already mentioned in the “erratum” (Linkov *et al.*, 1999) referred to above. This is now our preferred classification scheme.

3. The *Crump* classification scheme was devised by Dr. K. S. Crump and colleagues in collaboration with Dr. J. Haseman (Crump, 1999). This system reduces the CBDS tumor codes to a total of 91 classes. This classification scheme is similar to that used by NTP in the TDMS database but adds a few classes for tumor sites and types not otherwise included.

4. The *tumor-bearing animals* classification is a simple one-class scheme that uses the individual animal, rather than the tumor, as the unit of analysis. We simply count the number of animals with at least one tumor in control and dosed groups.

5. The *total tumors* classification combines all tumors into a single group. For a given control or dose group in a bioassay, all tumors in all animals are combined regardless of site and multiple tumors in a single animal all contribute to the total.

In practice, complications arise in deriving the total number of tumors in a given number of animals because not all animals in a dose group are examined for tumors at all sites so that the denominator (as in number of tumors/number of animals) differs within a study. We must make a correction to our analytical procedure to account for this. We start with tumors in our standard classification (102 sites). For every group of animals, the number of tumors for each site in the standard classification was counted. The number of tumors in each experimental group was then normalized to correspond to a number of examined animals (*n*) of 50. If for example, the classification scheme had only two tumor classes, a group of 50 animals had 10 tumors of class 1, but only 23 animals had been examined, and 13 tumors of class 2, for which 49 had been examined, then the number of “total tumors” is calculated as follows $(10/23) * 50 + (13/49) * 50 \approx 35$ total tumors in a standardized group of 50 animals. The values of “total tumors” so

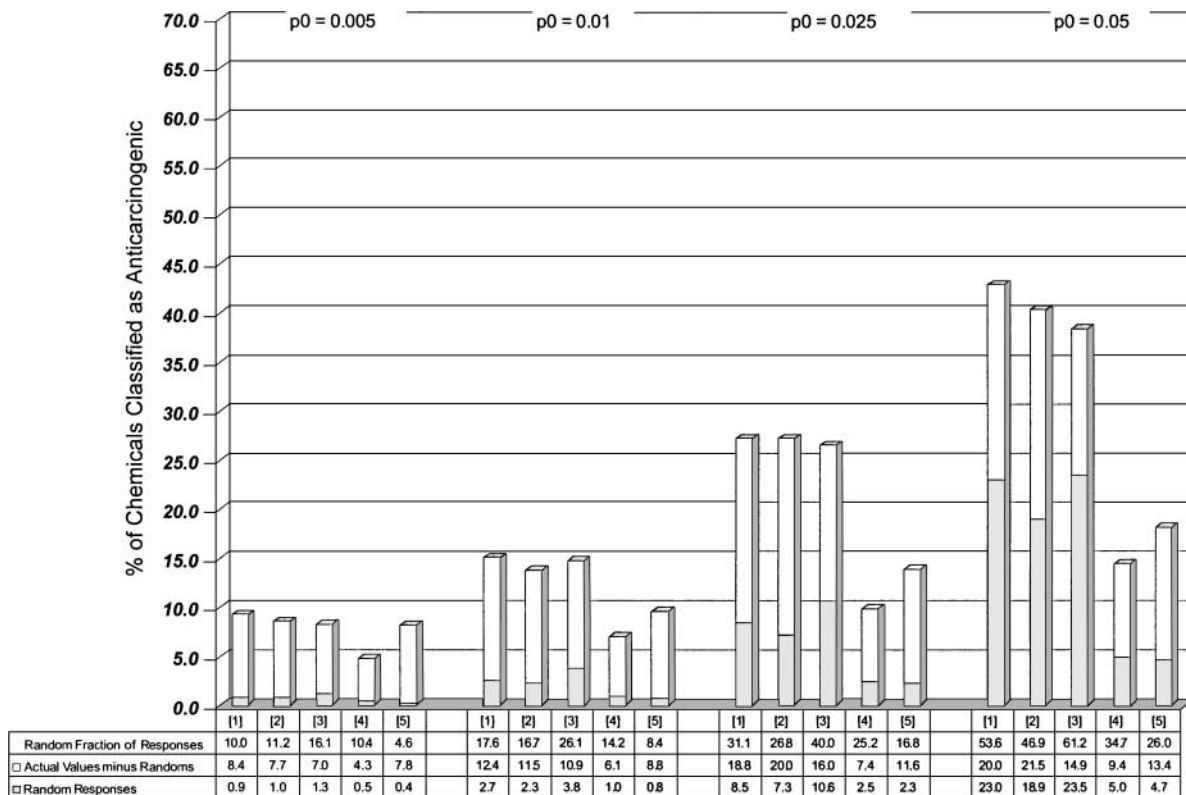


FIG. 1. Percentage of chemicals in the database classified as anticarcinogenic in male mice at different levels of P_0 for each classification system: [1] standard (old) classification; [2] modified (preferred) standard; [3] Crump classification; [4] tumor-bearing animals; and [5] total tumors. The shaded portion of each bar reflects the estimated random response with the New Monte Carlo for that sex, species, classification, and P_0 .

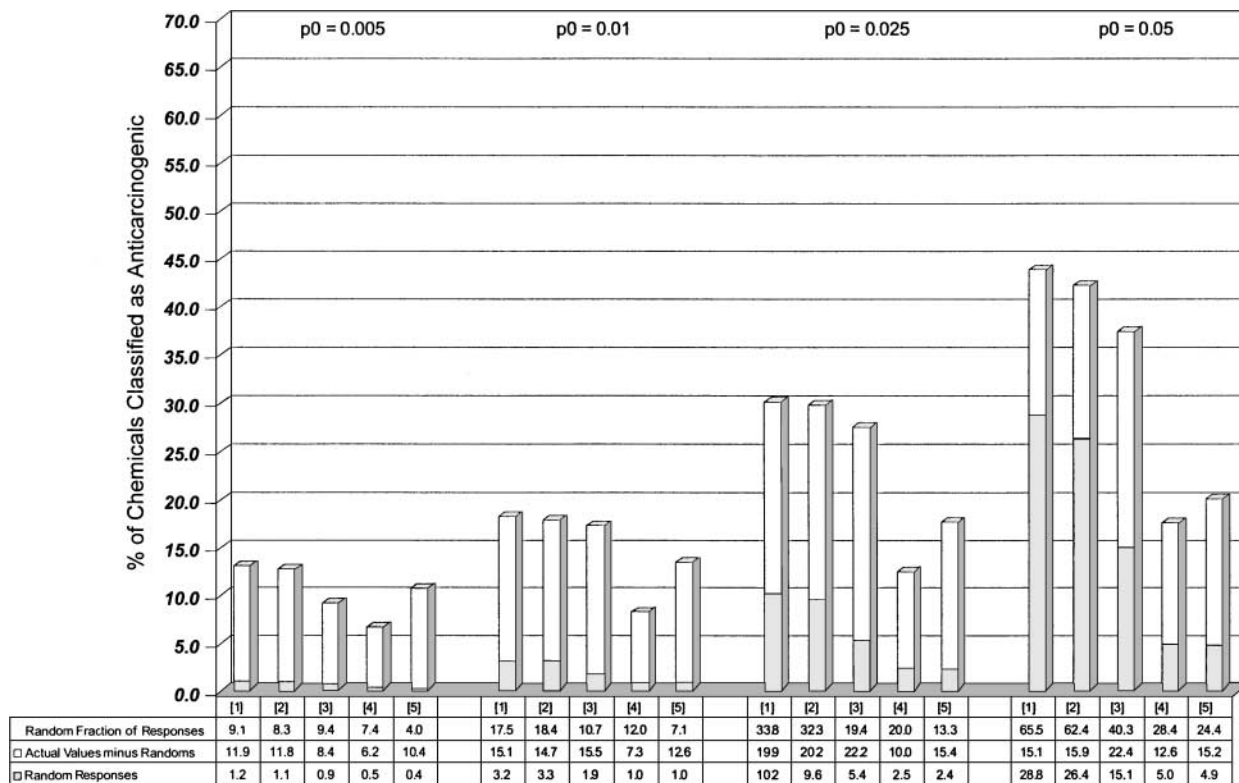


FIG. 2. Percentage of chemicals in the database classified as anticarcinogenic in female mice at different levels of P_0 for each classification system: [1] standard (old) classification; [2] modified (preferred) standard; [3] Crump classification; [4] tumor-bearing animals; and [5] total tumors. The shaded portion of each bar reflects the estimated random response with the New Monte Carlo for that sex, species, classification, and P_0 .

normalized were used as the input data for the evaluation of responses.

Response Evaluation

A chemical is classified as anticarcinogenic if there is a significant decrease of tumors in any class, of sites and tumor types, in a group of rodents upon dosing. We recognize the factors other than statistical significance play a role in interpretation of bioassay tumor responses (Haseman and Elwell, 1996) but with a large database and varying classification schemes, we used solely statistical measures for evaluating tumor dose response. Statistical significance was decided using Fisher's exact test and the Cochran-Armitage trend test according to the criteria in Table 1. The value of P_0 in this table is varied throughout the analysis. This approach is the same as that used in many of our earlier papers.

Random Effects

As has been pointed out by Haseman and Johnson (1996) and Linkov *et al.* (1998a) an increase or decrease in tumor rate is expected for some chemicals as

a purely random fluctuation in background tumor rates although, of course, we cannot tell which individual chemical is thereby spuriously labeled carcinogenic or anticarcinogenic. That random fraction was first estimated by a simple Monte Carlo calculation as described in Linkov *et al.* (1998a) with an assumption that the tumor rates in any class is independent of the rate in any other. In this paper we extend the model to include a correlation in tumor rates. The responses in a specific tumor type were generated randomly, but instead of assuming independence as in our previous study, we take into consideration empirical data on all possible correlations among the tumor types. If a correlation between two groups is great, we consider these two groups as one for the purposes of the Monte Carlo calculation.

TABLE 1

	Fisher exact test (group pairs)	Cochran-Armitage dose trend test
Anticarcinogen	$P < P_0$ for any one pair or $P < 2P_0$ for two pairs	$P < P_0$

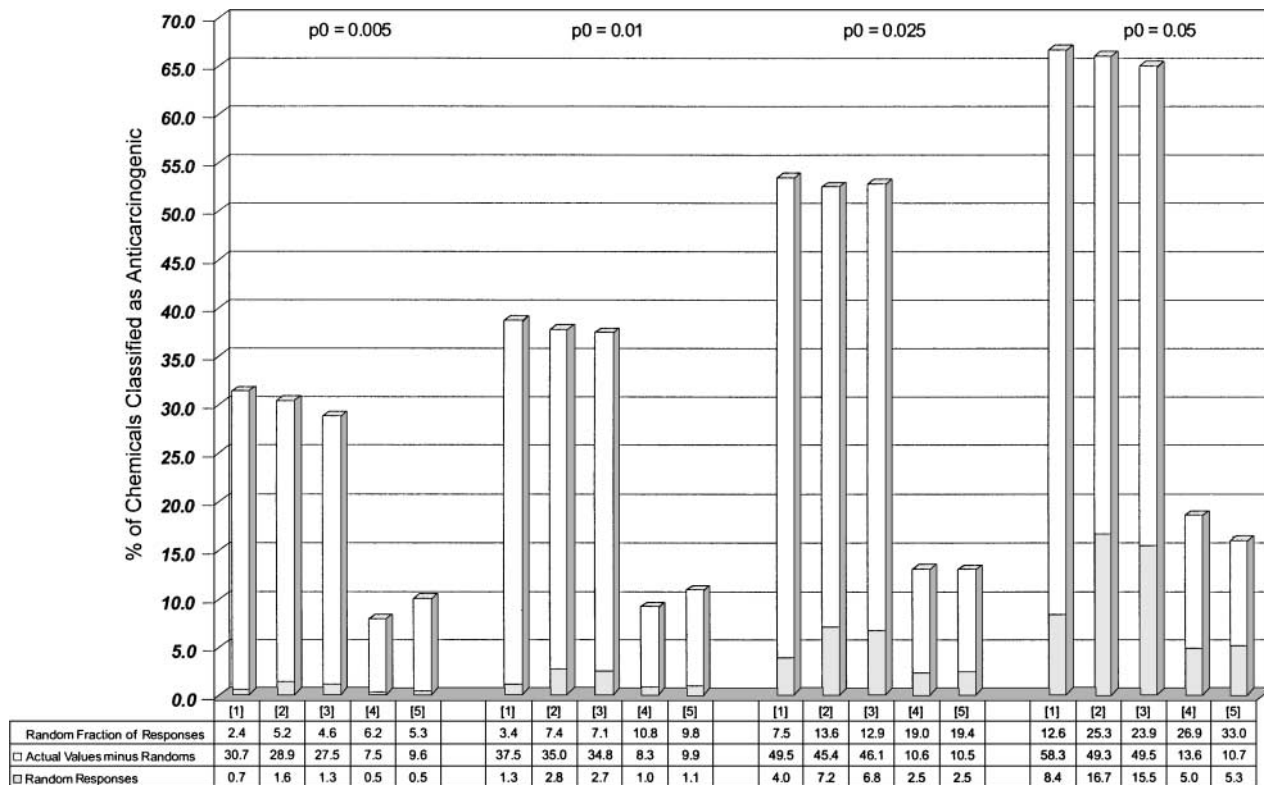


FIG. 3. Percentage of chemicals in the database classified as anticarcinogenic in male rats at different levels of P_0 for each classification system: [1] standard (old) classification; [2] modified (preferred) standard; [3] Crump classification; [4] tumor-bearing animals; and [5] total tumors. The shaded portion of each bar reflects the estimated random response with the New Monte Carlo for that sex, species, classification, and P_0 .

Detailed description of this simulation procedure is beyond the scope of this article and is available at <http://phys4.harvard.edu/~wilson/tumorgrouping/extra.html> (Wilson, 2000).

RESULTS AND DISCUSSIONS

Figures 1–4 compare the fraction (expressed as a percentage) of chemicals that would be judged anticarcinogenic in the four bioassay test groups (male and female mice and rats) using the five different classification schemes and four different values of P_0 , 0.005, 0.01, 0.025, and 0.05. The fraction of anticarcinogens is almost the same for each of the first three schemes. But the fraction drops appreciably when the single class schemes 4 and 5 are used.

Each classification scheme results in a different number of chemicals being classified as anticarcinogenic. This is shown in the pie graph of Fig. 5. Twenty-nine chemicals are found to be anticarcinogens in male mice (MM) with $P < 0.005$ using the old classification scheme (1), 27 using the modified scheme (2), and 26 using the Crump scheme (3). But of these only 13 chemicals are common to each scheme. These common chemicals are listed in Table 2.

The contribution of false-positive (random) effects to findings of anticarcinogenicity is shown as a dash line in Fig. 6. This estimate was done under the assumption that the tumor rate at each site is independent (Linkov *et al.*, 1998a). A similar assumption was also made by others (Haseman and Johnson, 1996) to estimate

TABLE 2
Chemicals Classified as Anticarcinogens by All the Schemes 1, 2, and 3

Chemical
2,7-Dichlorodibenzo- <i>p</i> -dioxin
2-Biphenylamine Hcl
8-Hydroxyquinoline
Azobenzene
Chlorodibromomethane
Guar gum
Hexylresorcinol
<i>N</i> -Butyl chloride
<i>N</i> -Phenyl-2-naphthylamine
<i>O</i> -Anisidine hydrochloride
<i>P</i> -Anisidine hydrochloride
Rotenone
<i>tert</i> -Butyl alcohol

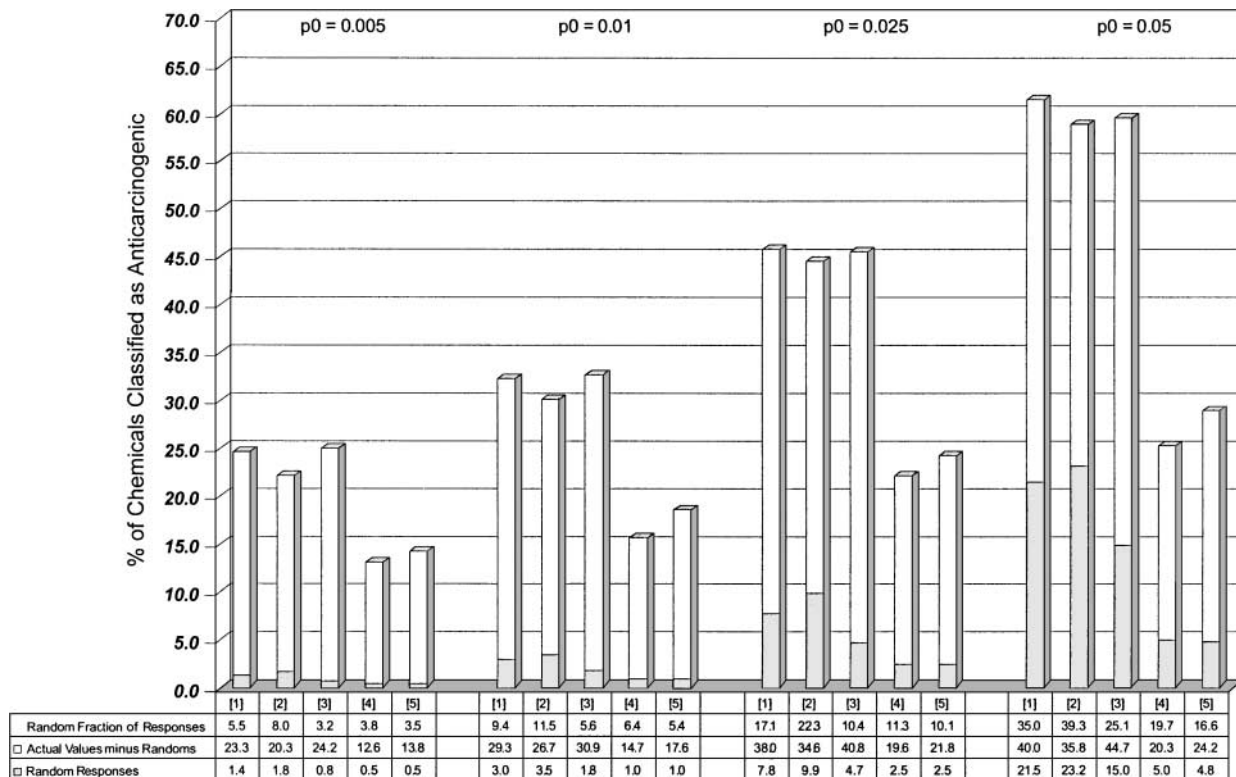


FIG. 4. Percentage of chemicals in the database classified as anticarcinogenic in female rats at different levels of P_0 for each classification system: [1] standard (old) classification; [2] modified (preferred) standard; [3] Crump classification; [4] tumor-bearing animals; and [5] total tumors. The shaded portion of each bar reflects the estimated random response with the New Monte Carlo for that sex, species, classification, and P_0 .

the false-positive effect rate. In this study, we have developed a model that incorporates the major correlations in tumor rate under simplified assumptions (shaded portion of the bar). This model takes the tumor

sites that are highly correlated or anticorrelated and treats them as one site. Clearly the model accounting for correlations predicts fewer positive responses due to random effects than the simple model. The figure also shows that at low values of P_0 (e.g., 0.005) there are few if any positive responses due to random effects. However, at less stringent levels of statistical significance (e.g., $P_0 = 0.05$) the number of random responses estimated using the simple model exceeds the number of measured real plus random responses. Nonetheless, at $P_0 = 0.05$ fully half of the findings of anticarcinogenicity are likely to be due to random variation in background tumor rates in classifications (1), (2), and (3). For the single class schemes (4) and (5) the random effects are correctly given by the simple Monte Carlo calculation and of course by the simple argument that at any value of P_0 , the fraction of all responses are due to random effects is P_0 .

As already noted in Linkov *et al.* (1999) there seem to be only small differences in the proportion of anticarcinogens found when using our “standard” classification scheme, and our “modified” scheme with liver tumors grouped together and pituitary adenomas grouped together. There are also only small changes in the proportion when using the Crump

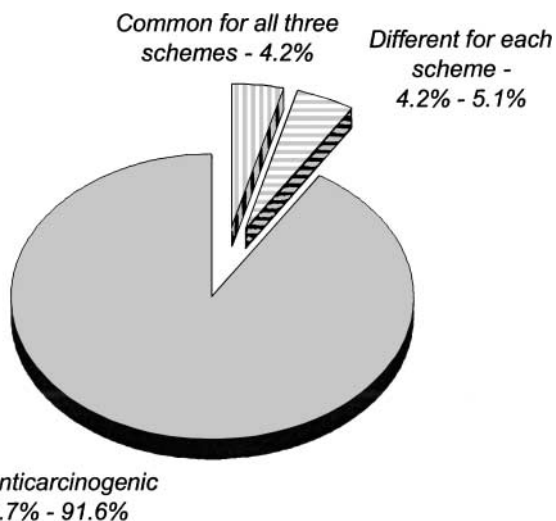


FIG. 5. Percentage of chemicals found to be anticarcinogens in one of the first three schemes (male mice at $P_0 = 0.005$).

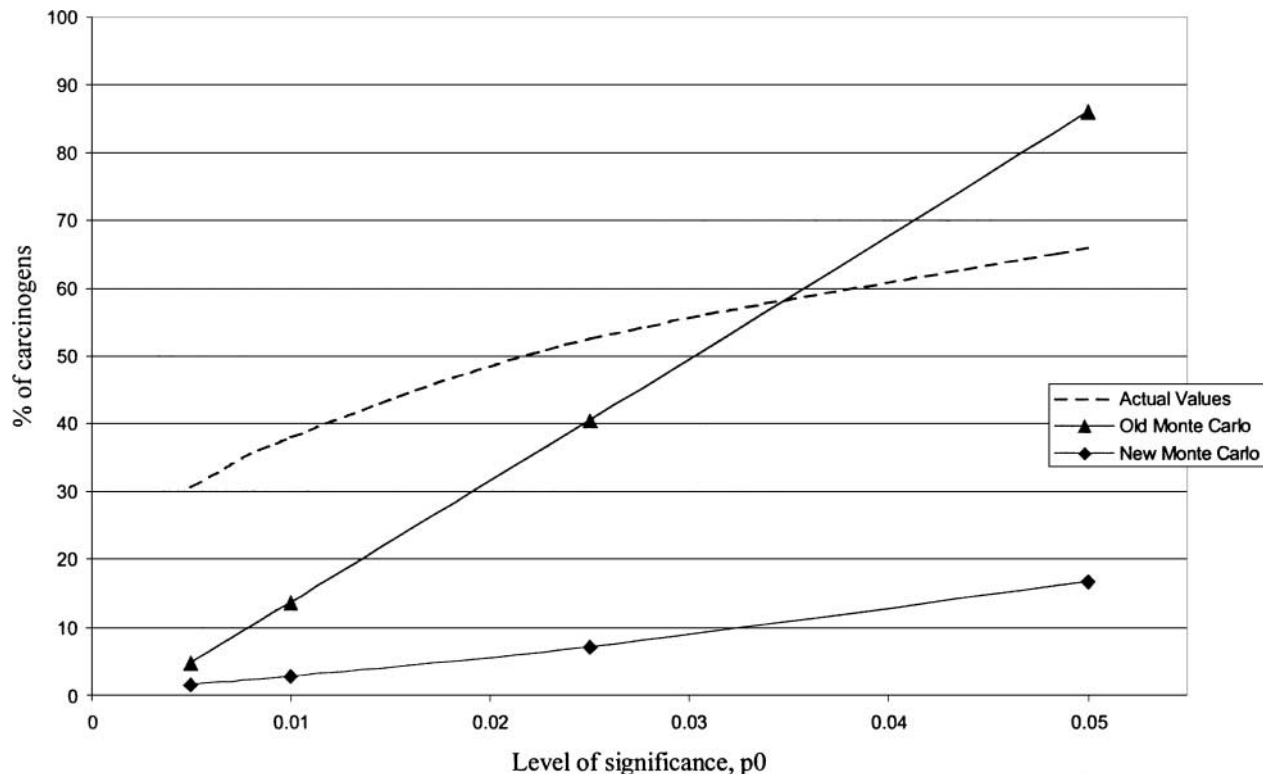


FIG. 6. Percentage of chemicals in the database classified as anticarcinogens in male rats at different values of P_0 using the modified classification (2) and fraction of chemicals classified as anticarcinogenic in Monte Carlo simulations of the bioassay in the absence of chemical effects using the unadjusted model (“old”) and the model accounting for significant correlations between tumor types (“new”).

scheme instead of one of ours. The smallness is probably due to the fact that the classes are very similar and use approximately the same number of tumor groups.

For any of the first three classification schemes the proportion of chemicals which show anticarcinogenicity purely by random effects, as judged by our simulation model, is a significant fraction of the chemicals found to be anticarcinogenic. At no level of significance did the random effects account for all of the cases of anticarcinogenicity, and at stringent levels of significance the fraction of random effects is less than half. Use of the classifications *tumor-bearing animals* or *total tumors* that integrate individual responses into a smaller number of tumor groups significantly reduces the number of chemicals judged to be anticarcinogenic. Much, but not all, of the reduction may be due to fewer random responses. The reduction is less if *total tumors* are used than if *tumor-bearing animals* are used.

We are able to detect several anticarcinogenic chemicals although the NTP rodent bioassay is not particularly sensitive to these effects. Anticarcinogenicity of a substance requires a reduction in tumors already present in the control group. Anticarcinogenicity can only be detected if there is an appreciable rate in the

control animals (typically in the range of 10–20% in our data). There are only a limited number of tumor types for which this occurs and these tend to be tumor types in the same class for each of the three different schemes.

We have also identified the fact that an anticarcinogenic response is more likely to be found if the number of animals with the tumor in the control group is higher than the number averaged over all studies (historical controls) as demonstrated in Fig. 7. This could indicate that many findings of anticarcinogenicity are due to upward fluctuations (greater than sampling statistics) in the rate of tumors in the “control” (undosed) animals. In these cases the tumor rate in the dosed groups is similar to the rate of tumors in the historical controls. On the other hand, a comparison with the control group may be the only appropriate way to evaluate responses because of other variations in rates of tumors in controls.

Integrative classifications address both of these shortcomings but create others. The sensitivity of the bioassay is reduced when measures like *tumor-bearing animals* or *total tumors* are used. Integrative classifications obviously exclude the common substances, which are carcinogenic at one site and anticarcinogenic at another. The tradeoffs between different methods of

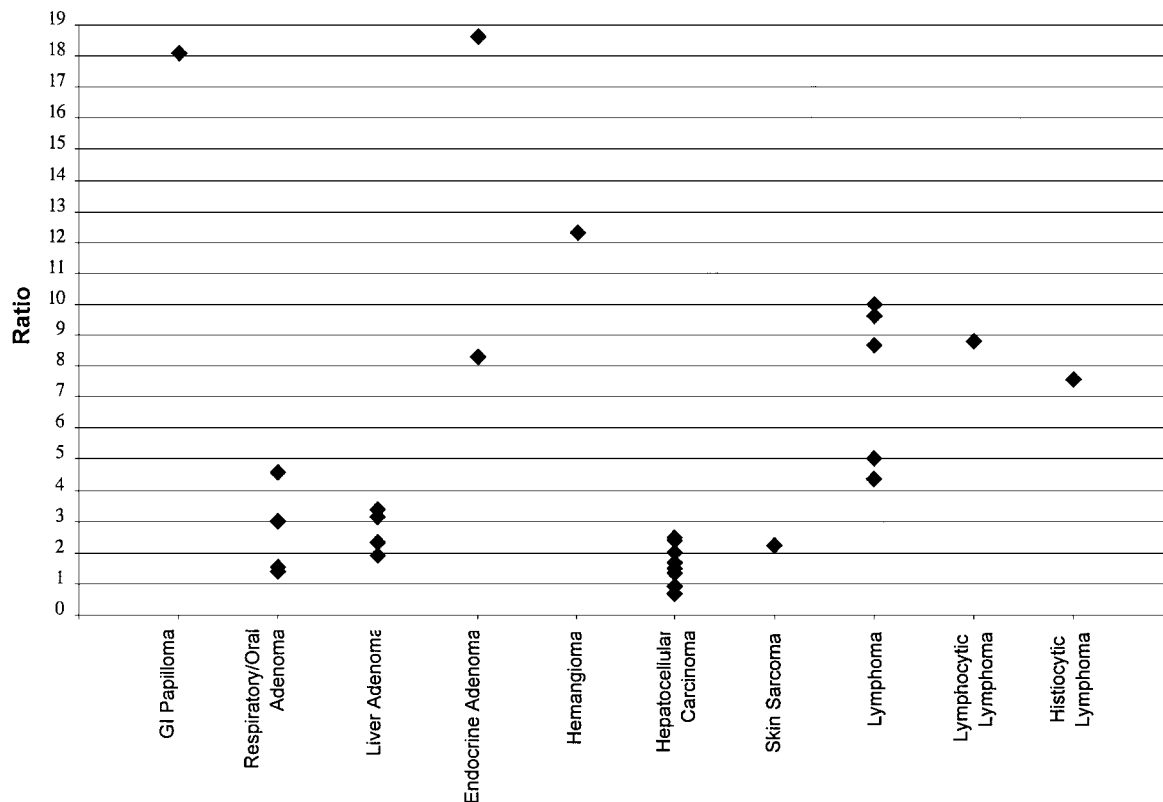


FIG. 7. Ratio (tumor rate in controls for experiments with anticarcinogenic responses)/(historical tumor rate in controls). Male mice only, modified standard classification.

classifying rodent bioassay tumors for risk assessment should be explicitly addressed in the context of the decision at hand. We have shown that the NTP bioassays are consistent with earlier papers showing that anticarcinogenesis is a common consequence of exposure to chemicals. The NTP bioassays are inherently unable to address the issue of whether, at low doses, anticarcinogenesis is even more common as suggested by the data on dioxin and 2-AAF. Rodent bioassays often play a major role in judgments about the carcinogenic potential of chemicals. If these judgments only focus on *increases* in tumor rates, or are otherwise faulty, attention and resources may be misplaced.

In recent years, policy analysts have been paying increasing attention to the risks caused by low doses of chemicals. The low-dose-linearity assumption based upon the work of Crump *et al.* (1976) seems logical and has been used as a default. But the sign of the slope of the dose-response curve is not specified and can be different for different tumors. Also unspecified is the point below which the dose can be considered to be low enough for the default to apply. We urge more bioassays where careful attention is paid to the low-dose (less than 1/10 of the maximum tolerated dose) region.

APPENDIX

The "Standard" Tumor Classification (102 Classes)

No.	Class
1	Skin, breast papilloma
2	Respiratory, oral papilloma
3	GI papilloma
4	Urinary, reprod. papilloma
5	Skin, breast adenoma
6	Respiratory, oral adenoma
7	Liver adenoma
8	GI adenoma
9	Urinary, reprod. adenoma
10	Pituitary adenoma
11	Endocrine adenoma
12	Skin, urinary adenoma
13	Reprod., endocrine adenoma
14	Tubular cell adenoma
15	Follicular, clear cell adenoma
16	Cortical adenoma
17	Skin, breast, liver cystadenoma
18	GI, urinary, reprod. cystadenoma
19	Endocrine cystadenoma
20	Acinar cell adenoma
21	Keratoacanthoma
22	Tubular adenoma
23	Interstitial cell tumor
24	Pheochromocytoma
25	Skin, breast fibroma

APPENDIX—Continued

No.	Class
26	Blood, bone fibroma
27	Fibroma, other sites
28	Lipoma
29	Leiomyoma
30	Endometrial stromal polyp
31	Fibroadenoma
32	Hemangioma
33	Osteoma
34	Hamartoma
35	Ganglioneuroma
36	Chromophobe adenoma
37	Skin, breast carcinoma
38	Blood, bone carcinoma
39	Lung carcinoma
40	Oral, GI carcinoma
41	Urinary carcinoma
42	Reproductive carcinoma
43	Pituitary carcinoma
44	Endocrine carcinoma
45	Brain carcinoma
46	Skin, breast papillary carcinoma
47	Lung papillary carcinoma
48	GI, urinary papillary carcinoma
49	Uterus, ovary papillary
50	Thyroid papillary carcinoma
51	Skin squamous carcinoma
52	Lung squamous carcinoma
53	Oral, GI squamous carcinoma
54	Urinary, reprod. squamous
55	Skin, GI basal cell carcinoma
56	Urinary transitional cell
57	Skin, breast adenocarcinoma
58	Lung adenocarcinoma
59	Oral, GI adenocarcinoma
60	Urinary, reprod. adenocarcinoma
61	Endocrine, brain adenocarcinoma
62	Islet cell carcinoma
63	Bile duct carcinoma
64	Hepatocellular carcinoma
65	Alveolar, broncheolar carcinoma
66	Chromophobe carcinoma
67	Tubular cell adenocarcinoma
68	Thyroid follicular cell carcinoma
69	Cortical carcinoma
70	Clear cell carcinoma
71	Adnexal, sebaceous carcinoma
72	Thymoma
73	Granulosa cell carcinoma
74	Interstitial cell carcinoma
75	Pheochromocytoma, malignant
76	Skin sarcoma
77	Other sites sarcoma
78	Blood, bone sarcoma
79	Liposarcoma
80	Leiomyosarcoma
81	Endometrial stromal sarcoma
82	Carcinosarcoma
83	Mesothelioma, osteosarcoma
84	Teratoma
85	Hemangiosarcoma
86	Granular cell tumor
87	Glioma
88	Oligodendroglioma
89	Astrocytoma

APPENDIX—Continued

No.	Class
90	Olfactory neuroblastoma
91	Neurofibrosarcoma
92	Lymphoma
93	Lymphocytic lymphoma
94	Histiocytic lymphoma
95	Mixed lymphoma
96	Malignant reticulosis
97	Leukemia
98	Myelomonocytic leukemia
99	Lymphocytic leukemia
100	Plasmacytic leukemia
101	Granulocytic leukemia
102	Monocytic leukemia

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