Prediction of Rodent Nongenotoxic Carcinogenesis: Evaluation of Biochemical and Tissue Changes in Rodents Following Exposure to Nine Nongenotoxic NTP Carcinogens

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We studied nine presumed nongenotoxic rodent carcinogens, as defined by the U.S. National Toxicology Program (NTP), to determine their ability to induce acute or subacute biochemical and tissue changes that may act as useful predictors of nongenotoxic rodent carcinogenesis. The chemicals selected included six liver carcinogens (two of which are peroxisome proliferators), three thyroid gland carcinogens, and four kidney carcinogens. We administered the chemicals (diethylhexyl phthalate, cinnamyl anthranilate, chlorendic acid, 1,4-dichlorobenzene, monuron, ethylene thiourea, diethyl thiourea, trimethyl thiourea, and *d*-limonene) to the same strains of mice and rats used in the original NTP bioassays (nine chemicals to rats and seven to mice). Selected tissues (liver, thyroid gland, and kidney) were collected from groups of animals at 7, 28, and 90 days for evaluation. Tissue changes selected for study were monitored for all of the test groups, irrespective of the specificity of the carcinogenic responses observed in those tissues. This allowed us to assess both the carcinogen specificity and the carcinogen sensitivity of the events being monitored. We studied relative weight, cell labeling indices, and pathologic changes such as hypertrophy in all tissues; a range of cytochrome P450 enzymes and palmitoyl coenzyme A oxidase in the liver; changes in the levels of plasma total triiodothyronine, total thyroxine, and thyroid-stimulating hormone (TSH) as markers of thyroid gland function; and hyaline droplet formation, tubular basophilia, and the formation of granular casts in the kidney. There were no single measurements that alerted specifically to the carcinogenicity of the agents to the rodent liver, thyroid gland, or kidney. However, in the majority of cases, the chemical induction of cancer in a tissue was preceded by a range of biochemical/morphologic changes, most of which were moderately specific for a carcinogenic outcome, and some of which were highly specific for it (e.g., increases in TSH in the thyroid gland and increases in relative liver weight in the mouse). The only measurements that failed to correlate usefully with carcinogenicity were the induction of liver enzymes (with the exception of the enzymes associated with peroxisome proliferation). Most of the useful markers were evident at the early times studied (7 days and 28 days), but no overall best time for the measurement of all markers was identified. The judicious choice of markers and evaluation times can aid the detection of potential nongenotoxic rodent carcinogens. *Key words*: kidney, liver, nongenotoxic carcinogenesis, thyroid. *Environ Health Perspect* 110:363–375 (2002). [Online 7 March 2002]
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The U.S. National Toxicology Program (NTP) is currently the main international source of rodent bioassay data, having reported on the carcinogenic activities of about 500 chemicals over the past 25 years (*1*). However, the disparity between the large number of chemicals considered worthy of evaluation for rodent carcinogenicity and the limited resources available for this task has led to consideration of a range of alternatives to the classical two-species rodent carcinogenicity bioassay protocol. Such alternatives extend from a variety of predictive techniques based on the chemical structure and genetic toxicity of chemicals (*2*) to the use of accelerated carcinogenicity bioassays based on the use of genetically modified rodents (*3*).

A serious complication in attempting to replace the standard bioassay is that different chemicals produce different tumors in different tissues and species of rodents, and it is unclear which of these tumor profiles define those carcinogens most likely to pose a commensurate hazard to humans. At the simplest level this is captured by the dichotomy between genotoxic and nongenotoxic rodent carcinogens. Such uncertainties become important when attempting to validate accelerated alternatives to the standard two-species rodent assay. For example, it is currently unclear whether any of the proposed alternatives should be required to predict the carcinogenicity of presumed nongenotoxic rodent carcinogens such as sodium saccharin and limonene, or whether their value should be judged in relation to their sensitivity to genotoxic (mutagenic) carcinogens such as dimethylnitrosamine, benzo[*a*]pyrene, and aflatoxin B$_1$. Pending resolution of this central question, there remains the regulatory need to identify, as efficiently as possible, both genotoxic and presumed nongenotoxic carcinogens to which humans may be exposed.

Methods probably already exist that can be used to anticipate, with an acceptable level of certainty, those rodent carcinogens that can noncontroversially be classified as genotoxic. These carcinogens are usually active in both rats and mice, and they are generally carcinogenic to more than one tissue. Consistent with these activities, they are usually overtly active in short-term *in vitro* and *in vivo* genetic toxicity assays. Designing alternative techniques for the prediction and recognition of such carcinogens will therefore be relatively easy, the only possible complication being the large number of candidate assays available for use.

The more difficult challenge is to devise reliable assays for the prediction and recognition of nongenotoxic rodent carcinogens (*4,5*). Apart from the difficulty of deciding the relevance of such rodent carcinogens to humans, there is the possibility that the species, sex, and tissue specificity of such carcinogens will be uniquely associated with chemically induced changes that occur only within the tissues subject to carcinogenesis. Approaches to this problem have been made by Tennant and colleagues (*6,7*), who proposed that a battery of different transgenic mice may be used to predict genotoxic and nongenotoxic carcinogens, and by Yamasaki (*8*), who has assessed the ability of the Syrian hamster cell transformation assay to predict all classes of rodent carcinogen *in vitro*. We designed the present study to evaluate the proposition that, for example, the unique carcinogenicity of ethylene thiourea to the rat thyroid gland and the unique carcinogenicity of limonene to the male rat kidney are associated with acute and subacute biologic changes occurring uniquely in those species and tissues subject to carcinogenesis by these agents (*9*).

We selected nine NTP carcinogens for study (Table 1); their carcinogenic status,

Table 1. Carcinogenicity data for the nine compounds used in these studies.

CAS No. (Reference) / Trivial name / Chemical name	Route (Gav or Feed)	Rats Low	Rats High	Mice Low	Mice High	Tumour site	Rat ♂ C	L	H	Rat ♀ C	L	H	Mice ♂ C	L	H	Mice ♀ C	L	H	♂R	♀R	♂M	♀M
[117-81-7] (13) DEHP, Diethylhexylphthalate	Feed[b]	0.6	1.2	0.3	0.6	L	2	2	10	0	4	16	18	29	38	0	14	34	P	P	P	P
						L*	6	12	24	0	12	26	28	52	58	2	24	36				
						L**	4	10	14	0	8	10	12	23	20	2	10	2				
[87-29-6] (12) CINN, Cinnamyl anthranilate	Feed	1.5	3.0	1.5	3.0	L							13	14	26	2	16	29	P	N	P	P
						L*							29	60	79	6	41	67				
						L**							16	46	53	4	25	38				
						K*	0	0	8													
						P*	0	0	7													
[115-28-6] (14) CEA, Chlorendic acid	Feed	0.062	0.125	0.062	0.125	L	6	10	2	0	6	10	18	35	40				CE	CE	CE	NE
						L*	10	44	46	2	10	32	28	47	54							
						L**	4	42	46	2	6	22	10	18	20							
						P**	0	8	12													
[106-46-7] (15) DCB, 1,4-Dichlorobenzene	Gav[c]	150 300 ♂ / 300 600 ♀	300 600 ♂	300	600	L							28	22	64	10	10	38	CE	NE	CE	CE
						L*							34	45	80	30	21	72				
						L**							10	27	32	20	13	42				
						K	2	6	14													
[150-68-5] (16) MON, Monuron	Feed	0.075	0.15	0.5	1	L	0	2	6										CE	NE	NE	NE
						L*	2	12	18													
						L**	2	12	14													
						K	0	2	16													
						K*	0	6	30													
						K**	0	4	14													
[96-45-7] (18) ETU, Ethylene thiourea	Feed	0.0083	0.025	0.033	0.1	L							27	38	90	4	58	94	CE	CE	CE	CE
						L							41	64	92	8	88	96				
						L**							22	32	18	4	66	28				
						TG*	2	26	74	6	16	61	2	2	58	0	4	76				
						PTG**							0	0	20	23	39	53				
[105-55-5] (10) DETU, N,N'-Diethylthiourea	Feed	0.0125	0.025	0.025	0.05	TG	0	2	23	0	2	17							CE	CE	NE	NE
						TG*	0	2	31	0	9	37										
[2489-77-2] (11) TMTU, Trimethylthiourea	Feed	0.025	0.05	ND?	0.1	TG				0	3	30							N	P	N	N
						TG*				0	3	49										
[5989-27-5] (17) LIM, d-Limonene	Gav	75 150 ♂ / 300 600 ♀	150 ♂ / 600 ♀	ND?	1,000	K	0	8	6										CE	NE	NE	NE
						K*	0	16	22													
						K**	0	8	16													
						K***	0	8	14													

Abbreviations: K, kidney (tubular cell); L, liver; P, pancreas (acinar cell); PTG, pituitary gland; TBA, tumor-bearing animals; TG, thyroid gland (follicular cell). Tumor codes: no symbol, carcinomas; *carcinomas + adenomas; **adenomas only, ***hyperplasia. Carcinogenic status as described in the NTP reports: CE, clear evidence for carcinogenicity; NE, no evidence for carcinogenicity. Carcinogenic status as described by Haseman et al. (44): N,negative for carcinogenicity; P,positive for carcinogenicity. Tumor data were summarized from the NTP Technical Reports cited; only the tumor data that led to the classification of the compound as carcinogenic are displayed.
[a]Males and females received identical doses unless indicated otherwise. [b]Dose levels for feeding studies are grams compound per 100 grams diet. [c]Dose levels for gavage studies are milligrams compound per kilogram body weight per day.

including an estimate of carcinogenicity at the lower of the two dose levels evaluated by the NTP, is summarized in Table 2. The chemicals selected include six liver carcinogens (two of which are peroxisome proliferators), three thyroid gland carcinogens, and four kidney carcinogens (including limonene, which is the most well-documented example of α2u-globulin-associated renal carcinogenesis in the male rat). The chemical structures of these agents, their general absence of genetic toxicity as reported in the appropriate NTP bioassay report, and the tissue and species specificity of their carcinogenic activities are all consistent with them being regarded as presumptive nongenotoxic carcinogens. Further, for the major tissues subject to carcinogenesis (liver, thyroid gland, and kidney), other investigators have described data consistent with the susceptibility of these tissues to nongenotoxic carcinogenesis (7).

We administered the chemicals at the same dose levels to the same strains of mice and rats used in the original NTP bioassays (10–18) (nine chemicals to rats, seven to mice). We collected tissues from groups of animals at 7, 28, and 90 days for evaluation of target tissues for those changes that have been proposed to represent an aspect of the carcinogenic process in these tissues, and consequently, to be considered as predictive of nongenotoxic carcinogenesis. One unique aspect of our study is that we monitored the tissue changes selected for study for all of the test groups, irrespective of the species and sex specificity of the carcinogenic responses observed in that tissue. This allowed unique insights into both the carcinogen specificity and the carcinogen sensitivity of the marker events being monitored.

The results generated by our investigations are extensive, and we believe that it is more helpful to present in detail only some of the experimental data. Our approach was to consider data in the context of addressing specific questions related to utility or specific tissue responses as predictors of nongenotoxic rodent carcinogenesis (Table 3). We assumed

from the outset that for a biologic change in a tissue to be considered useful for the prediction of nongenotoxic carcinogenesis in that tissue, it would have to be clear, unequivocal, and specific to the carcinogens. To that end, the test data have not been analyzed for statistical significance. In practice, that means the "Discussion" section of this paper is concerned only with self-evident results. Clearly, that enabling decision may have obscured subtle tissue changes of possible significance to the carcinogenic process, but such changes were not considered relevant to the stated aims of the study. To compensate for this decision, the complete database is available from the authors (compact disc) to enable other interested investigators to scrutinize and assess statistically the results.

Materials and Methods

Chemicals

Monuron (MON, 99% pure), ethylene thiourea (2-imidazolidine ethione; ETU, 98% pure), diethyl thiourea (DETU, 98% pure), R(+)-limonene (d-limonene; LIM,

97% pure), diethylhexyl phthalate (dioctyl phthalate; DEHP, 99% pure), and 1,4-dichlorobenzene (DCB, 99% pure) were obtained from Aldrich Chemical Co., (Gillingham, Dorset, UK). Chlorendic acid as the anhydride (CEA, > 99 % pure) was obtained from Aldrich Chemical Co. and was hydrolyzed before use. Trimethyl thiourea (TMTU, > 98% pure) was obtained from Tokyo Chemical Industries Inc. via Fluochem Ltd. (Glossop, Derbyshire, UK). Cinnamyl anthranilate, (CINN, > 99% pure) was synthesized by Lancaster Synthesis (Morecambe, Lancashire, UK).

Animals

Fischer 344 rats (6–7 weeks old on arrival) were obtained from Harlan UK (Bicester, Oxfordshire, UK) and were allowed approximately 2 weeks acclimatization. B6C3F$_1$ mice (4–5 weeks old on arrival) were also obtained from Harlan UK and were allowed approximately 4 weeks acclimatization. We performed animal studies in accordance with the UK "Animals (Scientific Procedures) Act." Animal care and procedures were carried out

Table 3. Questions about predicting nongenotoxic carcinogenesis in the rodent addressed in the present study.

Liver markers	To what extent are the following events associated specifically with liver carcinogenesis in the rat and mouse?
	Liver weight increases
	Liver cell hypertrophy and hyperplasia
	Increases in liver cell labeling index
	PCoA oxidase induction
	Total cytochrome P450 induction
	Induction of CYP 1A1, 2B1/2, 3A1, and 4A1
Thyroid gland markers	To what extent are the following events associated specifically with thyroid gland carcinogenesis in the rat and mouse?
	Thyroid gland weight increases
	Follicular cell hypertrophy and hyperplasia
	Increases in thyroid cell labeling index
	Changes in total plasma T$_3$ and T$_4$ levels
	Compensatory changes to plasma TSH levels
Kidney markers	To what extent are the following events associated specifically with kidney carcinogenesis in the male rat?
	Kidney weight changes
	Kidney pathology
	Increases in kidney cell labeling index
Time of measurement	What is the optimum time for measuring those changes that appear to be associated with carcinogenesis?

Table 2. Qualitative conclusions of the carcinogenicity of the nine test agents.

	Liver				Thyroid				Kidney			
	Rat		Mouse		Rat		Mouse		Rat		Mouse	
Test agent (reference)	Male L H	Female L H	Male L H	Female L H	Male L H	Female L H	Male L H	Female L H	Male L H	Female L H	Male L H	Female L H
DEHP (13)	+* +	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –
CINN (12)	– –	– –	+ +	+ +	– –	– –	– –	– –	– +	– –	– –	– –
CEA (14)	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –
DCB (15)	– –	– –	+* +	– +	– –	– –	– –	– –	+* +	– –	– –	– –
MON (16)	+* +	– –	– –	– –	– –	– –	– –	– –	+* +	– –	– –	– –
ETU (18)	– –	– –	+ +	+ +	+ +	+ +	– +	+* +	– –	– –	– –	– –
DETU (10)	– –	– –	– –	– –	–* +	+ +	– –	– –	– –	– –	– –	– –
TMTU (11)	– –	– –	NS	NS	– –	–* +	NS	NS	– –	– –	NS	NS
LIM (17)	– –	– –	NS	NS	– –	– –	NS	NS	+ +	– –	NS	NS

Abbreviations: H, high dose; L, low dose; NS, not studied. Conclusions were based on the NTP bioassays; the low-dose classifications were provided by J. Haseman of the NTP.
*Haseman prefaced his conclusion with the word "probably."

according to in-house standards; all animals were housed in wire mesh cages. In the original NTP studies, animals were housed in solid cages with bedding; although housing animals in wire cages may remove contaminants from the animals' environment, we considered the caging difference to be of only minor significance. The temperature was controlled at 22° ± 3°C, humidity was controlled at 30–70%, and a 12hr/12hr light/dark cycle was maintained. Animals received PCD diet (Special Diet Services Ltd, Witham, Essex, UK), either untreated or combined with compound. Diet and water were available *ad libitum.*

Studies

Rats and mice were exposed to chemicals for 7, 28, or 90 days either in the diet (DEHP, CINN, CEA, MON, ETU, DETU, and TMTU) or by oral gavage (DCB and LIM). TMTU and LIM were not administered to mice. DEHP, CINN, CEA, MON, ETU, DETU, and TMTU were also administered for 365 days as satellite studies designed to study the time course of the changes in more detail. The results of those studies are not described here, except for the tumor incidences that were pertinent to the present results. Chemicals were homogeneously incorporated into the diet. Dietary analysis, carried out using methods specified in the NTP reports (10–18), confirmed that homogeneity and the target concentrations had been achieved. Dietary administration was continuous from the start of the studies until termination. We monitored food consumption weekly. Oral gavage was carried out daily, 5 days/week, except for the final 7 days of the studies (when minipumps were implanted) when oral gavage was carried out on all 7 days. Animals were terminated 24 hr after the final dose.

For determination of DNA synthesis (labeling indices), we implanted animals with osmotic minipumps (Rats: Altzet 2ML1; mice; Altzet 2001; both from Charles River UK Ltd, Margate, Kent, UK) containing bromodeoxyuridine (BRDU; 15 mg/mL in phosphate-buffered saline, pH 7.4) 7 days before termination. Animals were anesthetized with halothane, minipumps were inserted subcutaneously on the flank, and the wounds were closed with surgical clips or sutures.

Animals were killed by exposure to carbon dioxide, and blood was collected by cardiac puncture. Organs (liver, kidney, and thyroid gland) were removed and weighed, and sections were taken. The tissues were then processed by standard histologic procedures to paraffin blocks. Sections were cut and stained with hematoxylin and eosin (H&E) for histopathologic examination by

standard procedures. Thyroid glands (rats only) were weighed after fixation in formol saline and subsequent separation from the trachea. Thyroid glands in mice were too small to be accurately dissected and weighed. Additional sections were taken for determination of cell labeling indices (percentages of cells in S phase) by BRDU incorporation using the method of Soames et al. (19).

We homogenized the remaining hepatic tissue with a teflon-glass homogenizer in SET buffer (0.25 M sucrose, 5.4 mM EDTA 20 mM Tris-HCl, pH7.4) and prepared peroxisomal fractions (20). Microsomal fractions were prepared by centrifugation of the postperoxisomal supernatant at 105,000 *g* for 1 hr. The pellets were resuspended in 1.15% KCl containing 20mM Tris HCl, pH7.4, and centrifuged again at 105,000 *g* for 1 hr. The subcellular fractions were resuspended in SET and stored at –70°C.

Assays

Liver. We used the method of Omura and Sato (21) to determine the cytochrome P450 content of liver microsomes. We determined isoenzyme profiles on microsomal fractions using SDS-gel electrophoresis and Western blotting according to the general method of Bars et al. (22), with chemiluminescence detection using the Western

Light kit (Tropix, Bedford, MA, USA). Antibodies to P450s 1A1, 2B1/2, and 3A1 were supplied by Oxygene (Dallas, TX, USA), and antibodies to P4504A1 were a gift from David Bell (University of Nottingham, England). Positive controls used for the isoenzyme profiles were microsomes from animals treated intraperitoneally for 4 days with standard P450 inducing agents (23): β-naphthoflavone (100 mg/kg), phenobarbitone (80 mg/kg), dexamethasone (50 mg/kg), and methylclofenapate (25 mg/kg). We analyzed Western blots by eye and scored increases in P450 isozyme profiles relative to the negative and positive controls as either no changes in any animals (relative to negative controls), one or more animals showing mild increases, or one or more animals showing moderate or marked increases (marked increases were categorized as being of similar intensity to the positive control). Generally, there was little variation in response between animals. Palmitoyl coenzyme A oxidase activity (PCoA) was determined on peroxisomal fractions (24) and protein was determined using the method of Lowry et al. (25).

Thyroid. Plasma was prepared by centrifugation of whole blood. We determined plasma thyroid-stimulating hormone (TSH), total thyroxine (T_4), and triiodothyronine

Table 4. Parameters measured as potential predictors of nongenotoxic carcinogenesis and criteria used to define a biologically significant change.

Parameter	No.[a]	Clearly positive	Equivocal result	Negative result
		Results[b]		
Liver				
Relative liver weight[c]	10	≥ 120%	110–119%	< 110%
Labeling index[c]	10	≥ 200%	150–199%	< 150%
Liver hypertrophy[d]	10	≥ 50% moderate–marked	≤ 50% moderate–marked	Minimal/none
PCoA oxidase induction[c]	5	≥ 250%	200–249%	< 200%
Total P450 induction[e]	5	≥ 120%	115–119%	< 115%
P450 1A1 induction[e]	3	Moderate–marked	Mild	No change
P450 2B1/2 induction[e]	3	Moderate–marked	Mild	No change
P450 3A1 induction[e]	3	Moderate–marked	Mild	No change
P450 4A1 induction[e]	3	Moderate–marked	Mild	No change
Thyroid				
Relative thyroid weight[c]	10	≥ 125%	115–124%	< 115%
Labeling index[c]	10	≥ 200%	150–199%	< 150%
Hypertrophy[d]	10	≥ 50% moderate–marked	≤ 50% moderate–marked	Minimal/none
Hyperplasia[d]	10	≥ 50% moderate–marked	≤ 50% moderate–marked	Minimal/none
Plasma total T_3[c]	5	≤ 70%	71–80%	> 80%
Plasma T_4[c]	5	≤ 70%	71–80%	> 80%
Plasma TSH[c]	5	≥ 200%	150–199%	< 150%
Kidney				
Relative thyroid weight[c]	10	≥ 110%	105–109%	< 105%
Labeling index[c]	10	≥ 200%	150–199%	< 150%
Hyaline droplet formation	10	≥ 50% moderate–marked	≤ 50% moderate–marked	Minimal/none
Tubular basophilia[d]	10	≥ 50% moderate–marked	≤ 50% moderate–marked	Minimal/none
Granular cast formation[d]	10	≥ 50% moderate–marked	≤ 50% moderate–marked	Minimal/none

The criteria are arbitary and were based on the data obtained with the parameters, our experience, and data from the literature.
[a]Number of animals analyzed per group. [b]Group mean results. [c]Changes expressed as percent of control values. [d]Changes were assessed by histopathologic examination. Percentages indicate numbers of animals showing effects of this severity, compared to the incidence in control animals; in all cases only minimal or no effects were seen in control animals. [e]One or more animals showed the change indicated relative to the control group (marked increases were categorized as being of similar intensity to the positive control group: P450 1A1, β-naphthoflavone; P450 2B1/2, phenobarbitone; P450 3A1, dexamethasone; P450 4A1, methylclofenapate).
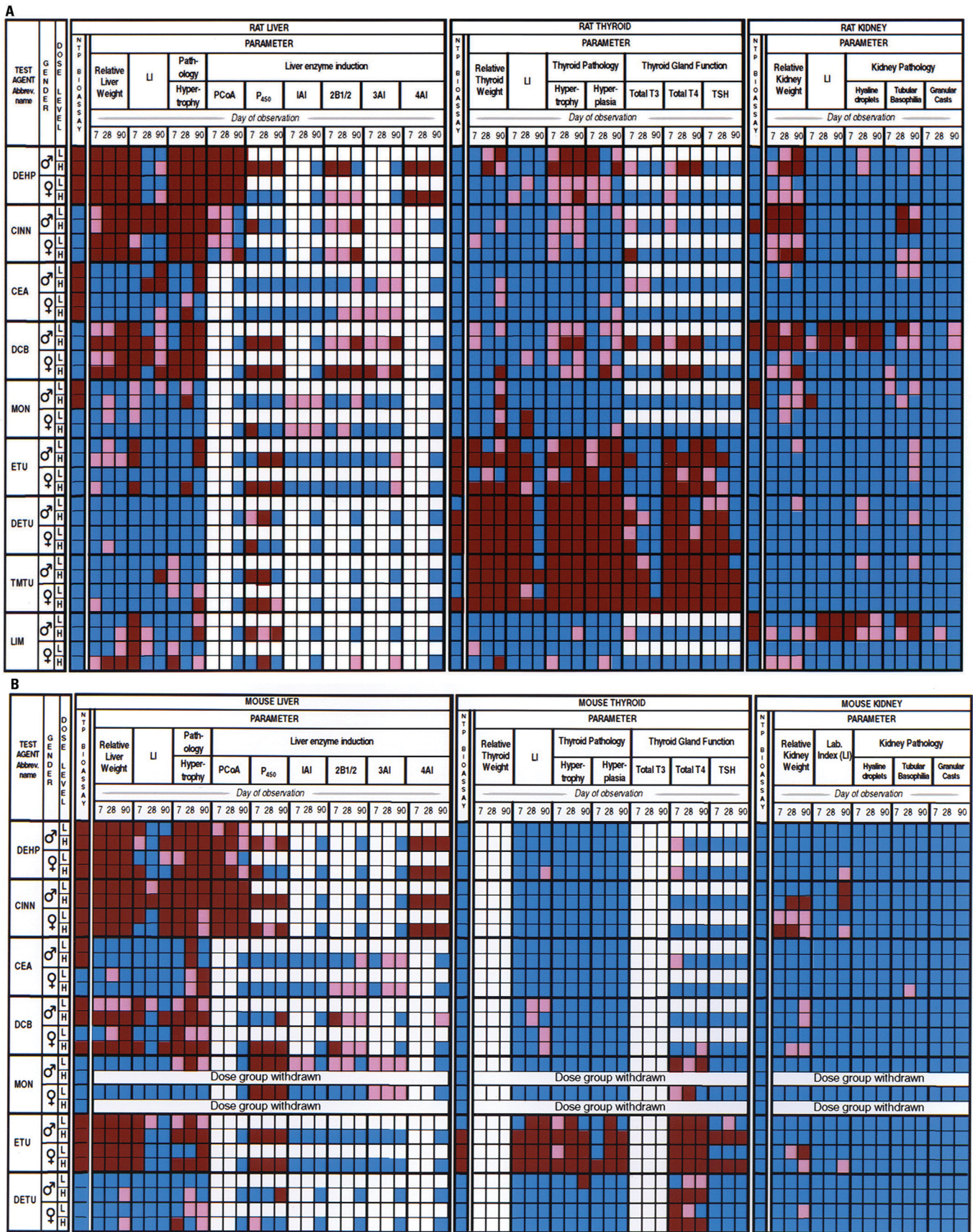
Figure 1. Schematic representation of the group mean results for the parameters given in Table 4 and the carcinogenicity results from the NTP bioassays for the rat (*A*) and the mouse (*B*). Abbreviations: H, high dose; L, low dose; LI, labeling index. Red, clearly positive results; blue, negative results; pink, equivocal result; white, parameter not determined.

(T$_3$) levels by radioimmunoassay using proprietary kits obtained from Amersham International (Buckinghamshire, UK; TSH) and Diagnostic Products Corp. (Los Angeles, CA, USA; T$_3$ and T$_4$). Plasma T$_3$ levels were not determined in mice because there was insufficient plasma for all assays.

Kidney. α2u-Globulin distribution was determined by immunohistochemistry as described by Stonard et al. (*26*).

Results

Body weight changes for rats and mice throughout most of the studies were within those previously observed in the NTP bioassays, and there were no clinical signs of toxicity for any of the compounds tested. The two exceptions to this were MON in the mouse, where unacceptable toxicity led to the discontinuation of studies in both males and females at the high doses (results not presented), and CINN, where unacceptable loss of body weight occurred in both species at the high dose. Food consumption for high-dose CINN was reduced, indicating poor palatability. The high dose of CINN was therefore reduced from 30,000 ppm to 22,000 ppm for both species.

The parameters listed in Table 4 were determined as potential predictors of nongenotoxic carcinogenesis in the liver, thyroid gland, and kidney. The criteria used to define clearly positive results, equivocal results, and negative results for each organ are also given. Data were not subjected to statistical analysis. We set the criteria after viewing the data for the parameters determined and based the criteria on our experience of interpreting changes with these parameters, literature data where possible (*27–29*), and the actual data obtained. We accept that our criteria are arbitary and that other workers may have chosen to use different criteria (e.g., a 4-fold increase in cell proliferation representing a positive result rather than the 2-fold increase we have chosen). A different set of results may have been obtained using a different set of criteria, but the overall trends discussed are unlikely to have changed. Numerical results are not presented for the parameters because of the volume of results. Instead, positive results, equivocal results, and negative results are presented in Figure 1 in color-coded schematic figures of the results for both species. The carcinogenicity results of the NTP bioassays are also presented in Figure 1 for comparison. Some time points and groups were not analyzed for certain parameters, for example, in cases where negative results were obtained for the high doses of compounds but the low doses were not analyzed. These are indicated in Figure 1.

The tumor incidences in animals exposed to DEHP, CINN, CEA, MON, ETU,

DETU, and TMTU for 1 year (in the satellite studies) are shown in Table 5. Where tumors were observed at this time point, the results were in accordance with those of the NTP except for TMTU, which is discussed below.

Rat

Liver. In the rat, liver weight and liver hypertrophy were clearly increased by

DEHP, CINN and DCB, as reported by others (*30–32*). MON, ETU, and LIM also gave weak increases in liver weight that were sometimes accompanied by liver hypertrophy. Increased liver weight after LIM administration has been shown previously (*33*). DETU, CEA, and TMTU gave little or no increases in liver weight, but CEA and TMTU did produce liver hypertrophy at later time points. Of the three rat

Table 5. Carcinogenicity of the chemicals after administration for 1 year as part of the present study.

Test agent	Sex, dose	Rat			Mouse		
		Liver	Thyroid	Kidney	Liver	Thyroid	Kidney
DEHP	Male						
	C	0	0	0	10	0	0
	L	0	0	0	30	0	0
	H	0	0	0	20	0	0
	Female						
	C	0	0	0	0	0	0
	L	0	0	0	11	0	0
	H	0	0	0	22	0	0
CINN	Male						
	C	0	0	0	10	0	0
	L	0	0	0	40	0	0
	H	0	0	0	10	0	0
	Female						
	C	0	0	0	0	0	0
	L	0	0	0	0	0	0
	H	0	0	0	10	0	0
CEA	Male						
	C	0	0	0	0	0	0
	L	0	0	0	11	0	0
	H	0	0	0	11	0	0
	Female						
	C	0	0	0	0	0	0
	L	0	0	0	0	0	0
	H	0	0	0	0	0	0
MON	Male						
	C	0	0	0	0	0	0
	L	0	0	0	10	0	0
	H	0	0	0	Withdrawn		
	Female						
	C	0	0	0	0	0	0
	L	0	0	0	0	0	0
	H	0	0	0	Withdrawn		
ETU	Male						
	C	0	0	0	0	0	0
	L	0	0	0	17	0	0
	H	0	90	0	75	100	0
	Female						
	C	0	0	0	0	0	0
	L	0	0	0	0	0	0
	H	0	20	0	100	86	0
DETU	Male						
	C	0	0	0	10	0	0
	L	0	0	0	0	0	0
	H	0	30	0	30	0	0
	Female						
	C	0	0	0	0	0	0
	L	0	0	0	0	0	0
	H	0	10	0	20	0	0
TMTU	Male						
	C	0	0	0			
	L	0	0	0			
	H	0	0	80			
	Female						
	C	0	0	0			
	L	0	30	0			
	H	0	100	0			

Abbreviations: C, control; H, high dose; L, low dose. Values shown are the percentage of tumor-bearing animals (combined adenoma and carcinoma incidence).

liver carcinogens (DEHP, CEA, and MON) in this group of chemicals, only the peroxisome proliferator DEHP showed an association between liver growth and hepatocarcinogenicity. A better association was observed between labeling index (at 7, 28, or 90 days) and hepatocarcinogenicity. DEHP, CEA, and MON all increased labeling index, although the time courses were different. The noncarcinogens CINN, DCB, ETU, TMTU, and LIM also gave increases in labeling index; only DETU had no effect. Increases in labeling index produced by some of these compounds have been shown previously (27,30,31,34).

All of the compounds tested gave increases in cytochrome P450, determined either as total (spectral) P450 or as specific isoforms (Western blots). Where the P450 induction profiles of compounds have previously been described (32,35,36), the results were as expected; for example, the peroxisome proliferator DEHP gave clear increases in total P450 and CYP 4A1 and some weak increases in CYP 2B1/2. In some cases, CEA, for example, total P450 was not increased, but there was weak induction of CYP 2B1/2 and CYP 3A1. This indicated a redistribution in the profile of P450s present in the liver. In other cases, such as ETU, DETU, and TMTU, the reverse occurred: total P450 was increased but there were no changes in the isozymes tested. It is probable that these chemicals induced a P450 isoform that was different from those tested.

We observed increases in PCoA with the peroxisome proliferators DEHP and CINN. Table 6 shows the quantitative changes in induction of PCoA. DEHP produced a much greater increase than CINN in the rat, correlating with the carcinogenicity of DEHP and noncarcinogenicity of CINN in this species. The increases in PCoA after DEHP and CINN treatment are as described previously (31,32).

Thyroid gland. In the rat thyroid, weight, hyperplasia, and hypertrophy were increased at most dose levels and time points by the three thyroid carcinogens tested: ETU,

DETU, and TMTU. However, specificity between low and high doses, and between sexes within the chemicals was not apparent. Low-dose DETU in male rats was not carcinogenic, and TMTU was only carcinogenic in high-dose female rats; similar profiles of effects for these three markers were observed across both sexes and groups. TMTU, however, when administered for 1 year, gave a different profile of thyroid gland tumors from those seen in the NTP studies, with increased tumor incidence in high-dose males and high- and low-dose females (Tables 1, 2, and 5). The results of all of the short-term markers correlate better with these results. ETU, DETU, and TMTU decreased total T_4 after 7 days of treatment. Effects on T_4 levels at later time points differed between these three compounds; for example, TMTU at both dose levels and sexes decreased T_4 after 28 and 90 days, whereas T_4 levels were unaffected in males given both doses of DETU at 90 days. TSH levels were generally increased when T_4 levels were decreased, in agreement with the known mechanism of feedback control in the thyroid (37). The thyroid labeling index usually paralleled the increased TSH levels; for example, all groups

with increased TSH at 7 days also had increased labeling indices. T_3 levels were decreased when T_4 levels were decreased, but changes in this parameter were not as closely paralleled by TSH and labeling index as T_4.

In the rat, the thyroid noncarcinogens DEHP and DCB produced clear decreases in T_4 levels in some groups, thyroid hypertrophy, and increased thyroid weight. There were no increases however in TSH levels or labeling index, although some thyroid hyperplasia was observed histopathologically. CINN decreased T_4 and T_3 levels after 7 days and produced some hypertrophy, indicating a weak effect on the thyroid. These compounds were also the most potent inducers of liver growth and P450 isoforms in the liver. Chemicals that produce these effects in the liver increase the clearance of thyroid hormones by increasing hepatic blood flow. Inducers of CYP 2B1/2 also induce the glucuronyl transferases responsible for the metabolism of T_4 and T_3 and hence increase their clearance (38). CEA, MON, and LIM gave some changes in the parameters tested for the thyroid gland, but there were no consistent patterns indicative of effects in the thyroid.

Table 6. The effect of DEHP and CINN on PCoA oxidase activity.

| Species | Sex/time | DEHP[a] | | CINN[a] | | Control values[b] |
		Low dose	High dose	Low dose	High dose	(nmol/min/mg)
Rat	Male					
	7 day	662	735	227	252	15.2 ± 7.5; 6.2 ± 0.7[c]
	28 day	467	916	218	241	11.8 ± 2.8; 18.6 ± 2.5
	90 day	611	1,036	161	187	12.4 ± 2.5; 20.0 ± 2.6
	Female					
	7 day	295	398	206	189	20.3 ± 5.3; 24.0 ± 2.6
	28 day	336	531	234	219	12.2 ± 8.0; 20.3 ± 1.7
	90 day	395	661	195	253	16.0 ± 2.4; 22.1 ± 1.7
Mouse	Male					
	7 day	211	325	419	513	17.2 ± 6.5
	28 day	258	332	426	422	22.9 ± 5.2
	90 day	225	249	311	341	17.7 ± 4.1
	Female					
	7 day	300	317	345	459	20.5 ± 3.3
	28 day	240	321	360	430	23.9 ± 4.1
	90 day	183	206	282	299	25.3 ± 5.2

[a]Values are expressed as percentage of control values. [b]Actual control values. [c]The first and second sets of figures refer to the control groups from separate rat studies with DEHP and CINN, respectively; in the mouse, studies with DEHP, CINN, and a common control group were run concurrently.
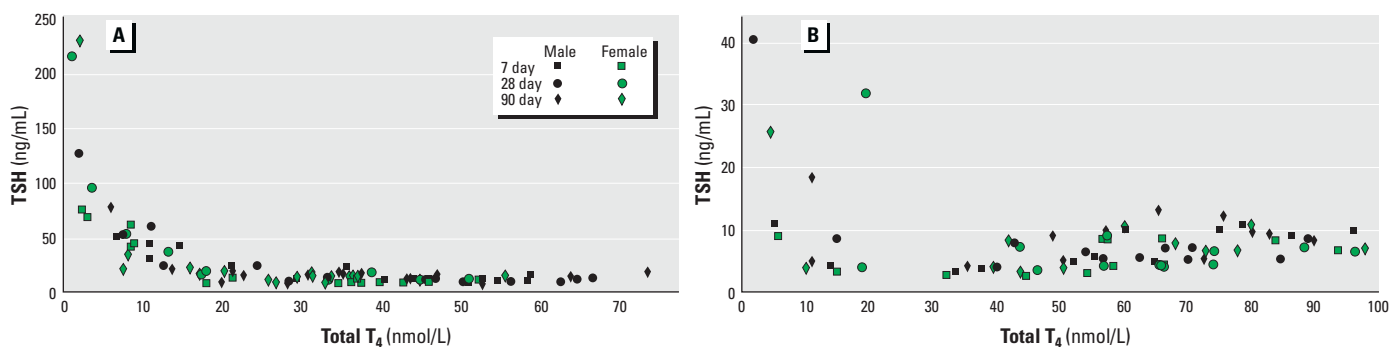

Figure 2. The relationship between (plasma) total T_4 and TSH in (*A*) the rat and (*B*) the mouse. Data points represent the group means for control groups and all nine (rat) or seven (mouse) compounds at both dose levels for all time points and both sexes.

We investigated the relationship between T_4 and TSH by plotting mean TSH versus T_4 levels for untreated and treated male and female rats for all of the chemicals and at all of the time points (Figure 2A). This relationship indicates that there is a threshold level of T_4 that must be reached before TSH begins to increase. In rats of both sexes, this appears to be approximately 20 nmol/L. Once this threshold is reached and TSH rises, DNA synthesis is thought to be stimulated in the thyroid, although there does not appear to be a simple quantitative relationship between TSH and labeling index (data not shown). In the cases of the thyroid carcinogens ETU, DETU, and TMTU, levels of T_4 decreased below the threshold of 20 nmol/L; therefore, the later events of increased TSH and labeling index, thyroid hyperplasia, and marked increases in thyroid weight followed. For the noncarcinogens affecting thyroid markers, plasma T_4, although decreased, did not fall below the threshold; therefore, the later events did not follow.

Kidney. In the rat, dose- and duration-related increases in kidney weights were produced by DEHP, CINN, DCB, MON, and LIM in both male and female animals. DCB, MON, and LIM are male kidney carcinogens, but it was notable that the greatest effects on kidney weight were produced by chemicals that also had the greatest effects on liver weight. The increases in kidney weight were more marked in males but were also observed in females. CEA, ETU, DETU, and TMTU had little or no effect on kidney weight. The rat kidney carcinogens DCB, MON, and LIM increased the labeling index in male rat kidney, although the time courses were different (Figure 3). MON increased the labeling index after 7 days only, whereas DCB and LIM increased the labeling index after 28 and 90 days. CINN (also a male rat kidney carcinogen) had no effect on labeling index in the rat, but at early time points this may have been due to decreased body weight gain due to nonconsumption of diet. Increased hyaline droplet formation in males was observed at all time points with DCB and LIM [as described by Hard et al. (39)], but not with MON or CINN. Immunohistologic staining of kidney sections from these groups confirmed that α2u-globulin was increased with DCB and LIM but not with MON or CINN (data not shown). Tubular basophilia was evident to some degree in all groups in male animals only, although the most marked changes were seen with CINN, DCB, and LIM. DCB and LIM were the only compounds that produced granular casts at the corticomeullary junction. The findings with DCB and LIM are in agreement with the known mechanism of action

of these compounds; that is, α2u-globulin accumulation and the subsequent changes associated with this (40).

Mouse

Liver. In the mouse liver, weight, labeling index, and liver hypertrophy were clearly increased by the hepatocarcinogens DEHP, CINN, DCB, and ETU. Some of the effects of DEHP, CINN, and DCB have been shown previously (30,31,41). CEA, MON, and DETU gave little or no increases in liver weight but did produce some hypertrophy, which was observed microscopically. In this species, CEA was carcinogenic in males only. The hepatocarcinogens DEHP, CINN, DCB, and ETU increased the labeling index at 7, 28, and 90 days, although CEA failed to increase the labeling index at any time point. The liver noncarcinogens MON and DETU had no effect on labeling indices.

All the chemicals tested gave increases in P450, determined either as total (spectral) P450 or as specific isoforms (Western blots). Induction of P450 by DEHP, CINN, and DCB was in agreement with previous work (31,42,43). We observed induction of CYP isoforms without increases in total P450 (CEA), and we also observed increases in total P450 without induction of the isoforms tested (ETU, DETU) as in the rat.

The peroxisome proliferators DEHP and CINN gave clear increases in PCoA in the mouse. Both of these hepatocarcinogens are known to be peroxisome proliferators in mice (27). Table 6 shows the quantitative changes in induction of the peroxisomal marker enzyme PCoA. The increases in this parameter correlate well with the carcinogenicity of CINN in mice and to some extent with DEHP, although here induction of PCoA was weaker.
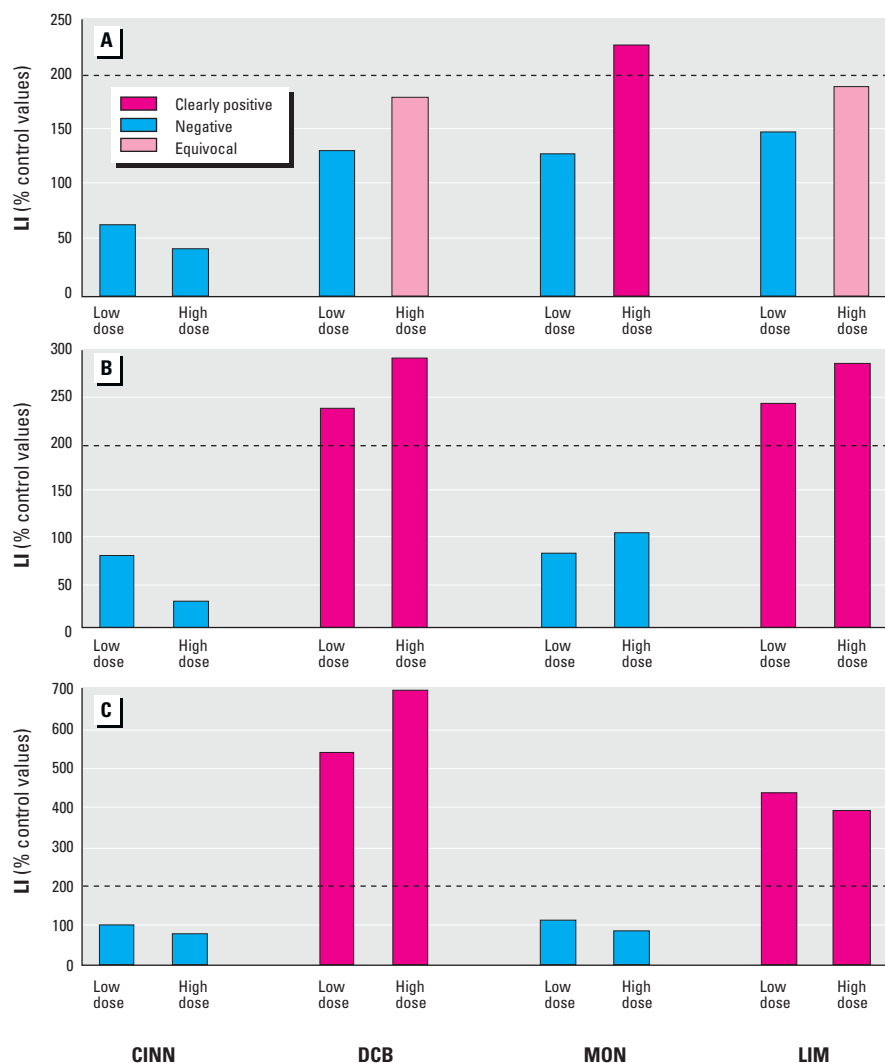

Figure 3. Labeling indices (LI) for NTP kidney carcinogens in male rat kidneys after (*A*) 7, (*B*) 28, and (*C*) 90 days. The colors of the bars represent the classification of the results as shown in Figure 1. The dashed line indicates the threshold for a clearly positive result for LI (≥ 200% of control as defined in Table 4). All test compounds were carcinogenic to the male rat kidney except for the low dose of CINN.

Thyroid. In the mouse, ETU was the only thyroid carcinogen tested. Decreased T_4, increased TSH, labeling index, and thyroid hypertrophy and hyperplasia, at all time points, were consistant with the dose and sex specificity for carcinogenicity in low- and high-dose males and high-dose females. The lack of effect of ETU on TSH in females given carcinogenic (low) doses however was not consistent with dose and sex specificity.

The noncarcinogens DETU and MON in the mouse produced clear decreases in T_4 levels, but these were not accompanied by increases in TSH levels, labeling index, or histopathologic changes. DEHP, CEA, and DCB gave occasional small decreases in T_4 without affecting TSH levels. The effects of DEHP and DCB on the thyroid were much less marked than those observed in the rat. The only chemical that was completely without effect was CINN.

As in the rat, we investigated the relationship between T_4 and TSH in mice by plotting mean TSH versus T_4 levels for untreated and treated male and female mice for all the chemicals and at all the time points (Figure 2B). The relationship is not as clear as in the rat, probably because only one chemical was a thyroid carcinogen. Nevertheless, Figure 2 indicates that there is

also a threshold level of T_4 in the mouse, which must be reached before TSH starts to rise. This also appears to be approximately 20 nmol/L. The same relationship between TSH, DNA synthesis, thyroid hyperplasia, and marked increases in thyroid weight is thought to exist in the mouse. As in the rat, the thyroid carcinogen ETU decreased T_4 levels below the threshold of 20 nmol/L and thus the later events followed. For the noncarcinogens affecting thyroid markers, plasma T_4, although decreased, did not fall below the threshold; therefore, the later events did not follow.

Kidney. None of the compounds were kidney carcinogens in the mouse. Increased kidney weights were observed with CINN, DCB, and ETU only. These compounds also produced marked liver growth in the mouse. CINN also increased the labeling index. Hyaline droplets, tubular basophilia, and granular cast formation were not seen in any mice.

Discussion

The present study was initiated to investigate, in a systematic way, whether biologic changes that have previously been associated with nongenotoxic rodent carcinogenesis could singly, or in concert, form the basis of a predictive strategy. To this end, we simulated as

far as possible the conditions of bioassay used by the NTP while defining the carcinogenicity of nine rodent carcinogens (44). During the early stages of this simulation, we evaluated a range of acute/subacute markers of presumed nongenotoxic rodent carcinogenesis. The markers used in this study have been reported by others for the rodent liver (27,28,30,31,41), kidney (9,26,33,39,40), and thyroid gland (9,37,38). The results of the study for individual markers are shown schematically in Figure 1. The extent to which the positive carcinogenicity classifications for the test chemicals are followed by the respective markers in the tissues sensitive to carcinogenesis become clear. Before analyzing the data in detail (according to the questions posed in Table 3), we will discuss four general conclusions of the study that influence the analysis.

First, gross inspection of Figure 1 reveals that the marker results are not random and that, in general, the acute/subacute tissue changes monitored are associated with carcinogenesis. A specific example of this is the good performance of the rat kidney markers for all of the chemicals tested, an effect enhanced by the essential totality of negative marker responses (blue entries in Figure 1) in the mouse kidney, corresponding to the absence of mouse kidney carcinogens in this study. However, a danger faced when analyzing the data in more detail is that the results may be fragmented to yield perhaps unjustified species- or chemical-specific conclusions regarding the predictive value of individual markers. Although this may be acceptable when supported by a mechanistic rationale, as with the use of enzymes associated with peroxisome proliferation in predicting peroxisome proliferator rodent liver carcinogens, it can be delusory when performed without such a guiding principle. An example of the latter would be to conclude that the bank of positive markers (red entries in Figure 1) associated with DEHP in the rat liver are predictive of its rat liver carcinogenicity, while ignoring the equally large bank of positive marker results in the rat liver for the rat liver noncarcinogen CINN. In contrast, both DEHP and CINN are carcinogenic to the mouse liver, activities that are supported by the bank of red liver markers seen for both chemicals in the mouse (Figure 1). This danger is particularly relevant to most of the earlier studies in this area in which acute/subacute "predictive" tissue responses of isolated carcinogens were monitored in isolated species and tissues without the concomitant evaluation of negative control agents. Similar conditional conclusions could be derived and then challenged for the responses in the rat to the three thyroid gland carcinogens ETU, DETU, and TMTU

Table 7. Prediction of carcinogenicity in the rat or mouse liver by organ-specific parameters.[a]

| Parameter | Rat Percent of data sets | | | | Mouse Percent of data sets | | | |
	7 day	28 day	90 day	n	7 day	28 day	90 day	n
Relative liver weight								
Accuracy[b]	72	64	58	36	88	88	84	26
Sensitivity[c]	40	40	40	10	82	82	82	17
Specificity[d]	85	73	65	26	100	100	89	9
Labeling index								
Accuracy	44	64	67	36	81	38	62	26
Sensitivity	40	10	20	10	71	6	41	17
Specificity	46	85	85	26	100	100	100	9
Liver hypertrophy								
Accuracy	64	64	56	36	69	92	69	26
Sensitivity	40	60	60	10	65	94	65	17
Specificity	73	65	54	26	78	89	78	9
PCoA oxidase induction								
Accuracy	ND	ND	82	22	ND	ND	50	18
Sensitivity			57	7			31	13
Specificity			93	15			100	5
Total P450 induction								
Accuracy	28	33	50	18	72	50	64	14
Sensitivity	20	20	20	5	14	21	78	9
Specificity	31	38	62	13	14	29	40	5
P450 isoform induction[e]								
Accuracy	ND	ND	61	18	ND	ND	64	14
Sensitivity			40	5			44	9
Specificity			69	13			100	5

Abbreviations: *n*, number of total number of data sets; ND, not determined.
[a]Prediction of carcinogenicity in the rat and mouse was assessed by counting numbers of data sets shown in Figure 1; a data set was defined as the carcinogenicity result (C) and parameter (P) results for a specific compound, species, sex, dose, and time point (e.g., one data set includes the carcinogenicity result and the liver weight result for male rats given low-dose DEHP). [b]The correct identification of carcinogenic activity for all chemicals (*50*) (i.e., Figure 1, C blue, P blue and C red, P red). [c]The proportion of carcinogenic responses correctly identified for carcinogenic chemicals only (*45*) (i.e., Figure 1, C red, P red). [d]The proportion of noncarcinogenic responses correctly identified for noncarcinogenic chemicals only (*45*) (i.e., Figure 1, C blue, P blue); equivocal results (Figure 1, pink) were taken to be negative for this analysis. [e]A positive result for any of the isoforms was taken as a positive for this parameter.

(Figure 1). The first overall conclusion is therefore that the predictive value of individual markers can sometimes be seminally modulated by focusing either on carcinogens and species in general, or on specific carcinogens in specific species.

The second general conclusion derives from consideration of the counterproposal, namely, that positive marker changes seen for a chemical classified as noncarcinogenic to a particular tissue indicate the presence of a carcinogenic potential for the agent in that tissue which was not realized during the course of the NTP carcinogenicity bioassay. Some credence for this possibility is afforded by the data available for TMTU. This compound was classified by the NTP as being carcinogenic only in the thyroid gland of female rats, and then only at the high dose (Tables 1 and 2). In the present study, the majority of the marker events in the rat thyroid gland gave positive results for TMTU, irrespective of the dose studied and the sex of rat evaluated (Figure 1). This would indicate that the thyroid gland markers studied were not specific for the carcinogenicity of TMTU, despite being predictive of the thyroid gland carcinogenicity of the high dose of TMTU to the female rat. However, in the satellite study in which rats were exposed to TMTU for 1 year, it was found that TMTU was carcinogenic to both high- and low-dose female rats and to high-dose male rats. This carcinogenic profile correlates well with the marker changes observed at the earlier time points (Figure 1). Obviously, the NTP carcinogenicity data must be maintained as the primary calibrant for this study, but the data derived here for TMTU suggests that the carcinogenic profile of a chemical is not absolute (4–7,45) and that, in fact, tissue changes seen for some chemicals classified as noncarcinogenic may be predicative of an as yet unrealized, or perhaps ultimately unrealizable, carcinogenic activity.

The third general conclusion relates to the second, and concerns the possibility that some tissue markers may be indicative of tissue changes necessary for carcinogenesis, but are insufficient for it when they occur alone. This would reduce the value of the markers but not render them useless. This possibility is illustrated by the changes in thyroid hormones observed (Figure 2) for the thyroid gland carcinogens ETU, DETU, and TMTU (Tables 1 and 2, Figure 1). From these data (Figure 2) it is clear that reduction of total T_4 levels to about 20 nmol/L are required before increases in TSH are observed, the latter being considered to be the event most predicative of the induction of thyroid gland hyperplasia and eventual carcinogenesis (37). A cascade is therefore indicated by these data in which reductions

in T_4 levels can be seen as contributing to the carcinogenic process, but being nonspecific for it until the reduction is sufficient to trigger an elevation in TSH levels. This concept severely complicates analysis of the database because markers may be prematurely neglected due to their failure to correlate fully with carcinogenesis in the affected tissue. Equally, some markers may be totally independent of the carcinogenic process, as probably indicated by the cytochrome P450 changes seen here in the rodent liver. Resolving these two possibilities requires judgment, itself open to preconception and unintentional bias.

The final general conclusion relates to the proposed validity of our decision to apply qualitative positive test criteria to our data, as opposed to conducting statistical analyses of the data. This decision is the same as that adopted by pathologists when assessing a tissue for chemically induced changes: the terms minimal, mild, moderate, severe being universally owned for this purpose (46). Similarly, the original NTP low-dose carcinogenicity bioassay results were concluded qualitatively for the present study by Joseph

Haseman of the NTP (Table 2). The qualitative test outcome criteria used here were based on our experience with the end points beyond this study (Table 4), in particular, knowledge of the magnitude of changes that could be considered beyond experimental variability and therefore likely to be chemically induced. The practice of these criteria is exemplified in Figure 3 for the four rat kidney carcinogens evaluated. Rigorous statistical analyses of those test data (Figure 3) may have influenced, challenged, or refined close calls such as the 7-day equivocal result for the high dose of LIM and the 7-day positive result for the high dose of MON. However, we concluded that the general sweep of the test results (Figure 1) would not have been substantially changed by statistical analyses, and it may even have been confused by positive statistical conclusions that failed to carry practical credibility. As noted earlier, the primary test data from this study will be made available to interested parties for statistical analysis.

Liver. The overall accuracy figures for the liver markers considered are shown in Table 7. The only high accuracy figures are for increases in the relative weight of mouse

Table 8. Prediction of carcinogenicity in the rat or mouse thyroid gland by organ-specific parameters.[a]

Parameter	Rat Percent of data sets				Mouse Percent of data sets			
	7 day	28 day	90 day	n	7 day	28 day	90 day	n
Relative thyroid weight								
Accuracy[b]	86	81	69	36	ND	ND	ND	
Sensitivity[c]	88	75	75	8				
Specificity[d]	86	82	68	28				
Labeling index								
Accuracy	89	81	78	36	100	96	96	26
Sensitivity	100	75	13	8	100	100	100	3
Specificity	86	82	96	28	100	96	96	23
Thyroid hypertrophy								
Accuracy	78	78	78	36	96	96	96	26
Sensitivity	75	88	100	8	67	100	100	3
Specificity	82	75	71	28	28	100	96	23
Thyroid hyperplasia								
Accuracy	81	83	86	36	92	96	100	26
Sensitivity	75	88	100	8	33	100	100	3
Specificity	79	82	82	28	100	96	100	23
Total T_3								
Accuracy	62	63	67	24	ND	ND	ND	
Sensitivity	50	13	13	8				
Specificity	69	88	94	16				
Total T_4								
Accuracy	79	63	66	24	72	78	78	18
Sensitivity	100	88	75	8	100	100	100	3
Specificity	69	56	63	16	67	73	73	15
TSH								
Accuracy	83	75	67	24	94	94	94	18
Sensitivity	88	63	25	8	67	67	67	3
Specificity	81	81	88	16	100	100	100	15

Abbreviations: *n*, number of total number of data sets; ND, not determined.
[a]Prediction of carcinogenicity in the rat and mouse was assessed by counting numbers of data sets shown in Figure 1; a data set was defined as the carcinogenicity result (C) and parameter (P) results for a specific compound, species, sex, dose, and time point (e.g., one data set includes the carcinogenicity result and the liver weight result for male rats given low-dose DEHP). [b]The correct identification of carcinogenic activity for all chemicals (50) (i.e., Figure 1, C blue, P blue and C red, P red). [c]The proportion of carcinogenic responses correctly identified for carcinogenic chemicals only (45) (i.e., Figure 1, C red, P red). [d]The proportion of noncarcinogenic responses correctly identified for noncarcinogenic chemicals only (45) (i.e., Figure 1, C blue, P blue); equivocal results (Figure 1, pink) were taken to be negative for this analysis. [e]A positive result for any of the isoforms was taken as a positive for this parameter.

liver following chemical treatment (≥ 84% accuracy). Overall, none of the markers correlate well with rat liver carcinogenicity, albeit the mouse figures are marginally better. Cytochrome P450 (total and isoenzymes) induction generally correlated poorly with carcinogenicity. The PCoA and the P450 4A1 induction data are somewhat artificial, given that these are specific enzymes associated with peroxisome proliferation, and only one rat and two mouse peroxisome proliferator liver carcinogens were included in the study (DEHP and CINN; Table 6). Nonetheless, the induction of these two enzymes was highly specific for these two chemicals in both rat and the mouse liver (enzyme induction was only determined for the high dose groups for the remaining seven chemicals). Overall, the best general markers of rodent liver cancer are increases in relative liver weight and cellular labeling index (particularly for the mouse) and liver hypertrophy. There was no optimum time for the measurement of these changes, albeit the early time point (7 days) was generally the best.

Thyroid gland. With the exception of T_3 and T_4 levels, all of the markers studied correlated well with thyroid gland carcinogenicity (Table 8). The low accuracy values for decreases in the levels of T_3 and T_4 are probably associated with the need to exceed a threshold reduction before increases in TSH are produced (see above and Figure 2). The accuracy of all of the markers would have

been increased if TMTU had been classified as a carcinogen in male rats (high dose) and female rats (low and high doses) instead of just for the high-dose female rats (as in the NTP study; see above and Table 5).

Kidney. Because no mouse renal carcinogens were included in this study, the bank of blue (negative results) for the mouse kidney markers (Figure 1) acts as an indicator of the high specificity of the positive marker responses seen for the four rat renal carcinogens evaluated (CINN, DCB, MON, and LIM; Table 9). As expected from earlier studies, increases in cell labeling indices (especially at the later sampling times) and hyaline droplet formation (particularly at the earlier sampling times) acted as good predictors of the carcinogenicity of DCB and LIM. These changes, which were specific to the affected male animals, are consistent with the α2u-globulin mechanism of male rat renal cancer induction. The absence of increases in kidney weight for animals exposed to LIM (and MON) is in contrast to the increases seen for animals exposed to DCB (and CINN), but there is no obvious explanation for these differences. The carcinogenicity of the remaining two rat renal carcinogens (CINN and MON) was poorly predicted by the markers evaluated. Relative kidney-weight increases for CINN were not specific to the affected males, and no such changes were induced by MON. Similarly, renal tubular basophilia was specific to the

affected male rat in the case of CINN but was not seen for animals exposed to MON.

Time of measurement. We observed that increases in relative tissue weight, independent of the carcinogenicity of the agent to that tissue, occurred from the earliest (7 days) to the latest times monitored here (90 days). With few exceptions (e.g., MON in the rat thyroid and DCB and LIM in the rat liver), increases in labeling index for all three tissues are most evident at the 7-day sampling period. Liver hypertrophy either occurs at all three sampling times or sporadically across the chemical test groups at the later two sampling times. There is no obvious correlation between these two patterns and rat liver carcinogenicity. Thyroid gland hypertrophy and hyperplasia correlate well at all time points with carcinogenicity, except in the case of DEHP in the rat. Among the liver enzyme induction effects seen, only those for PCoA and P450 4A1 (enzymes associated with peroxisome proliferation) correlated usefully with carcinogenicity, and then only when the two peroxisome proliferator carcinogens were considered. In these cases the changes were apparent at all of the sampling times evaluated. Increases in TSH correlated well with thyroid gland carcinogenicity, and these increases were evident primarily at the earlier sampling times. Decreases in total T_3 and T_4 were less specific for carcinogenicity and also occurred primarily at the earlier sampling times. The time course of changes in the rat kidney is in accordance with the mechanism of α2u-globulin-induced nephropathy in which hyaline droplet formation is the earliest event, followed by tubular basophilia, and then granular casts, which were an "equivocal" finding with DCB and LIM in this study.

The importance of cell proliferation and apoptosis. There is some controversy over the merit of determining transient increases in cell proliferation (as determined in this study) versus sustained increases, the latter of which have been suggested by some to be necessary to drive carcinogenesis. It is well known from initiation–promotion studies in which animals are treated with a mutagen and a subsequent regimen that induces cell proliferation that the relatively transient induction of cell replication associated with these procedures effectively promotes an already mutated population of cells to affect an enhanced number of tumors, or shortens the latency for tumor appearance (47). It is also apparent that the more rounds of replication that occur, as in sustained cytotoxin-induced cell replication, the more chances there are of inducing mutations in a previously nonmutated cell population or of inducing additional mutations in the population. Therefore, although it is clear that sustained cell replication carries an

Table 9. Prediction of carcinogenicity in the rat or mouse kidney by organ-specific parameters.[a]

Parameter	Rat Percent of data sets 7 day	28 day	90 day	n	Mouse Percent of data sets 7 day	28 day	90 day	n
Relative kidney weight								
Accuracy[b]	86	78	81	36	96	92	92	26
Sensitivity[c]	43	29	43	7	–	–	–	0
Specificity[d]	97	90	83	29	96	92	92	26
Labeling index								
Accuracy	83	92	92	36	100	100	92	26
Sensitivity	14	57	57	7	–	–	–	0
Specificity	100	100	100	29	100	100	92	26
Hyaline droplet formation								
Accuracy	89	89	86	36	36	100	100	26
Sensitivity	43	43	29	7	–	–	–	0
Specificity	100	100	100	29	100	100	100	26
Tubular basophilia								
Accuracy	81	86	89	36	100	100	100	26
Sensitivity	0	43	43	7	–	–	–	0
Specificity	100	97	100	29	100	100	100	26
Granular cast formation								
Accuracy	81	81	81	36	100	100	100	26
Sensitivity	0	0	0	7	–	–	–	0
Specificity	100	100	100	29	100	100	100	26

n, number of total number of data sets. [a]Prediction of carcinogenicity in the rat and mouse was assessed by counting numbers of data sets shown in Figure 1; a data set was defined as the carcinogenicity result (C) and parameter (P) results for a specific compound, species, sex, dose, and time point (e.g., one data set includes the carcinogenicity result and the liver weight result for male rats given low-dose DEHP). [b]The correct identification of carcinogenic activity for all chemicals (*50*) (i.e., Figure 1, C blue, P blue and C red, P red). [c]The proportion of carcinogenic responses correctly identified for carcinogenic chemicals only (*45*) (i.e., Figure 1, C red, P red). [d]The proportion of noncarcinogenic responses correctly identified for noncarcinogenic chemicals only (*45*) (i.e., Figure 1, C blue, P blue); equivocal results (Figure 1, pink) were taken to be negative for this analysis. [e]A positive result for any of the isoforms was taken as a positive for this parameter.

enhanced risk of developing cancer in the target tissue, an acute wave of replication, as can be seen with DEHP in the studies of Marsman et al. (*48*), is also associated with a carcinogenic response in the target tissue, the liver. Also, elevated rates of cell replication are not always associated with a carcinogenic response in the affected tissue (*49*). An interesting case is provided by chloroform. This chemical induced regenerative cell replication in the liver and kidney of B6C3F$_1$ and BDF1 mice, but the patterns of tumor response were very different between the two strains and were reflective of differing cytotoxicity as well as the genetic backgrounds and target organ and sex sensitivities (*49*). Thus, cell replication data can provide a useful aid to the prediction of nongenotoxic carcinogens, but such data must be used with care. We did not determine the rates of apoptosis in addition to cell proliferation because of the logistics of the current experimental design. However, assessment of apoptotic cell rates for those systems in which correlations between cell replication and cancer did not exist would be a rational way to test hypotheses regarding the effect of enhanced apoptosis on cancer outcome.

Conclusions

There is no single acute or subacute measurement that will alert specifically to the eventual appearance of chemically induced tumors of the rodent liver, thyroid gland, or kidney. The carcinogenic status of a chemical can therefore only be determined by reference to lifetime carcinogenicity bioassay data (*2,44*). Even so, it must be noted that the carcinogenic status of a chemical is not itself an absolute entity, as seen for TMTU in the present study in comparison to the NTP bioassay, and as noted for a range of other chemicals evaluated in accelerated transgenic rodent bioassays (*5–7,45*). However, in the majority of cases, the chemical induction of cancer in a tissue is preceded by a range of biochemical and morphologic changes, most of which are moderately specific for carcinogenicity and some of which are highly specific for it. Examples of the latter are provided by increases in TSH in the thyroid gland, the induction of hyaline droplets in the rat kidney, and increases in relative liver weight in the mouse. The only measurements that failed to correlate usefully with carcinogenicity were the induction of liver enzymes (with the exception of the enzymes associated with peroxisome proliferation: P450 4A1 and PCoA). The majority of the useful markers are evident at the early times studied (7 and 28 days), but we identified no overall best time for all markers. Therefore, we concluded that the judicious choice of markers and evaluation times can aid the detection of

potential nongenotoxic rodent carcinogens. To a large extent the choices made will be influenced by the class of chemical under study, which is prescribed in some situations (analogue development), but not in others (routine screening).

One of the more interesting findings of this study is the lack of alerts to the carcinogenicity of CEA in the liver of male and female rats and female mice. Marked increases in both liver carcinomas and liver adenomas were seen in all three of these test groups. Although there were some late-occurring increases in hypertrophy and labeling index in the rat liver and late-occurring liver hypertrophy in the mouse liver, these effects were not specific for carcinogenicity across the four test groups (male and female rats and mice).

Finally, the greatest strength of the present study is the ability to monitor changes occurring in tissues not subject to carcinogenesis by the test agents studied. This ability enabled severe qualifications to be applied to some of the markers that would have appeared to correlate well with carcinogenicity if only carcinogens for the tissues evaluated had been studied. For example, the success of liver weight changes, labeling index, and liver hypertrophy in the "prediction" of the rat liver carcinogenicity of DEHP has to be qualified once similar responses are seen for the rat liver noncarcinogen CINN (Figure 1). This further endorses the need for the adequate study of negative control agents in assay-validation studies.

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