From:	Jennifer Wagar
To:	P65Public Comments
Cc:	Alan Katz
Subject:	NOIL - furfuryl alcohol
Date:	Wednesday, September 30, 2015 9:28:38 AM
Attachments:	Response re OEHHA proposed listing of Furfuryl Alcohol 9-30-2015.pdf

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Dear Ms. Michelle Robinson,

On behalf of Illovo Sugar Ltd. ("Illovo") and its USA representative Harborchem LLC, IFC North America Inc., Pennakem LLC, TransFurans Chemicals BVBA and Agriguard Company LLC, attached please find comments on the Notice of Intent to List Furfuryl Alcohol, the comment period for which was extended to 30 September 2015.

If you have any questions, please contact Alan Katz at 703-754-0248 x111 or alan.katz@toxcel.com.

Kind regards,

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September 30, 2015

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RE: Response to Proposed Listing of Furfuryl Alcohol

Dear Ms. Robinson,

On July 31, 2015, OEHHA issued a notice of intent to list furfuryl alcohol as known to the state to cause cancer under the Safe Drinking Water and Toxic Enforcement Act of 1986 (also referenced as "Prop 65"). On behalf of Illovo Sugar Ltd. ("Illovo") and its USA representative Harborchem LLC, IFC North America Inc., Pennakem LLC, TransFurans Chemicals BVBA and Agriguard Company LLC, this letter discusses why furfuryl alcohol does not meet the "sufficient evidence" criteria for listing under Title 27, §25306 (e).

Summary

Furfuryl alcohol does not meet the criteria for "sufficient evidence" of cancer as set forth in Title 27, §25306. Furfuryl alcohol was listed because a committee within one Agency recognized as an authoritative body (US EPA) reviewed a study conducted by the National Toxicology Program (NTP) with two rodent species and a single route of exposure (inhalation) and classified the chemical as "likely to be a human carcinogen." However, the NTP study authors themselves reported only that there was "some" evidence of carcinogenic activity of furfuryl alcohol in male F344/N rats and male $B6C3F_1$ mice, and suggested that the maximum tolerated dose may have been exceeded in the studies.

The NTP study has been publicly available since 1999, and we are unaware of any other authoritative body that has reviewed the results and has come to the same conclusions as the review group within the EPA. Other toxicologists reviewing the studies have noted that in the rat, changes in nasal pathology were observed at all dose levels but neoplastic changes were only observed in males at the highest dose; this dose was also associated with increased mortality and decreased bodyweight gain and is considered to have exceeded the maximum tolerated dose. In the mouse, the occurrence of renal adenomas and carcinomas were restricted to only the 32 ppm (highest concentration) group along with associated pathological changes indicative of renal tubular injury. Since there is no counterpart to rodent chronic progressive nephropathy in humans, renal tumors in rodents resulting from chemical enhancement

of this spontaneous rodent pathological syndrome are not considered as a relevant indication of human risk.

Reviews by Authoritative Bodies and Expert Groups

The listing of furfuryl alcohol based on the EPA report, “Furfural and Furfuryl Alcohol: Report of the Cancer Assessment Review Committee (CARC)” does not meet the “sufficient evidence” criteria in Title 27, §25306 (reproduced below).

Title 27, §25306 (e) (2) *Sufficient evidence of carcinogenicity exists from studies in experimental animals. For purposes of this paragraph, “sufficient evidence” means studies in experimental animals indicate that there is an increased incidence of malignant tumors or combined malignant and benign tumors in multiple species or strains in multiple experiments (e.g., with different routes of administration or using different dose levels), or, to an unusual degree, in a single experiment with regard to high incidence, site or type of tumor, or age at onset.*

Review by the Cancer Assessment Review Committee (CARC)

The CARC assessment is based on studies conducted by NTP (1999) in the rat and mouse via the inhalation route; this is a route of exposure which is relevant to some industrial and agricultural uses but of less relevance to consumer exposure.

The referenced NTP study included only two species (mouse and rat), three dose levels, one route of administration (inhalation), and gave an inconsistent site of tumor occurrence between species. Therefore, the NTP study was insufficient for making a cancer determination based on the first part of §25306 (e) (2).

OEHHA’s intent to list appears to be based on the second part of §25306 (e) (2) because the 31 July notice states that EPA found that furfuryl alcohol causes increases in <u>rare</u> malignant nasal tumors in male rats and <u>rare</u> renal carcinomas and combined carcinomas and adenomas in male mice.

While the CARC recognizes the treatment-related nature of the tumors they do not characterize them as ‘rare’. The CARC report conclusions concerning furfuryl alcohol state:

> *In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005), the CARC classified* ***furfuryl alcohol as “Likely to Be Carcinogenic to Humans.”*** *This determination was based on the following:*
>
> *(i) Treatment-related nasal tumors (adenomas, carcinomas and/or squamous cell carcinomas observed in male rats. ;*
> *(ii) Treatment-related kidney tumors (adenomas, carcinomas and/or combined adenomas/carcinomas observed in male mice;*

In the body of the report, the CARC did not identify any of the nasal effects in the NTP furfuryl alcohol rat study as being rare; they have been observed with a number of substances which are known to be irritant, but are an uncommon background finding in untreated animals. Likewise, with regard to the mouse kidney tumors, the CARC noted that the presence of kidney tumors is rare in historical controls; however, the CARC report did not state whether the tumor type was a rare occurrence in treated mice such as in this study, for which multiple renal step sections were evaluated.

The CARC assessment considered that furfuryl alcohol was '***Likely to Be Carcinogenic to Humans'.*** At the doses used there was an effect in rodents; however, the following additional information must be taken into account with regard to human relevance.

Lack of genotoxicity

The CARC report reviewed a battery of genetic toxicology assays deemed acceptable for regulatory purposes, and summarized them saying*, "The data indicate that furfuryl alcohol is not mutagenic in bacteria and does not cause chromosome aberrations or SCE induction in mammalian cells. These in vitro data are supported by the results of whole animal studies showing that furfuryl alcohol was not clastogenic, aneugenic or genotoxic in mouse bone marrow, cytogenetic, micronucleus or SCE assays."* Thus, the CARC concluded that furfuryl alcohol does not present a genotoxic or mutagenic concern. Therefore the mode of action for tumour development is non-genotoxic and the result of some other mode of action.

Findings limited to dose levels which may have exceeded the Maximum Tolerated Dose (MTD)

Whereas the CARC stated that the doses used in the NTP study were adequate and not excessive, we maintain that the high dose likely exceeded the maximum tolerated dose in both the rat and mouse studies. Findings at these dose levels may be confounded by excessive toxicity.

A Principal Reviewer of the NTP study (1999) commented that "more attention should be paid to the 14-week rat study results in setting exposure concentrations for the 2-year study, in that **32 ppm appeared to exceed the maximum tolerated dose**" (emphasis added). This was also evident from the fact that the survival in both sexes of rats was significantly lower in the highest (32 ppm) treated group when compared to the controls. In fact, no male rats in the high concentration group survived to termination, and survival in female rats was decreased from 52 % (control) to 32 % (32 ppm).

Further, the strikingly high incidence of corneal degeneration in female mice exposed to the highest concentration (3/49, 1/49, 4/49, 26/50; at 0, 2, 8, and 32 ppm, respectively), supports a conclusion that the highest exposure level was excessive in this species as well.

Non-genotoxic mode of action in the rat

Regarding the nasal lesions in the rat study, it is considered that repeated local tissue damage at the portal of entry over time leads to an adaptive proliferation of cells and subsequent tumor formation (Weiler, 1997) resulting from chronic continued exposure. The non-neoplastic lesions of furfuryl alcohol including increased inflammation, hyperplasia of the lateral wall, atrophy and metaplasia of the olfactory epithelium, and hyperplasia of the respiratory epithelium are similar to those described for other nasal toxicants, demonstrating the likelihood of a similar, threshold mode of action.

The table below from the NTP study clearly shows that a number of adaptive responses and pathological processes are occurring at the site of entry.

	Chamber Control		2 ppm		8 ppm		32 ppm	
Male								
Number Examined Microscopically	50		50		50		50	
Suppurative Inflammation[a]	3	(1.0)[b]	6	(1.5)	17**	(1.7)	44**	(2.1)
Glands, Hyperplasia	0		0		22**	(1.0)	49**	(2.3)
Lateral Wall, Hyperplasia	1	(1.0)	49**	(1.5)	50**	(2.4)	50**	(3.5)
Lateral Wall, Squamous Metaplasia	1	(1.0)	1	(1.0)	8*	(1.1)	33**	(1.3)
Olfactory Epithelium, Atrophy	1	(1.0)	12**	(1.1)	47**	(1.8)	50**	(2.4)
Olfactory Epithelium, Hyaline Degeneration	42	(1.3)	48	(1.5)	50**	(2.6)	47	(2.7)
Olfactory Epithelium, Fibrosis	0		1	(1.0)	26**	(1.0)	40**	(2.0)
Olfactory Epithelium, Hyperplasia	0		1	(1.0)	42**	(1.0)	40**	(1.8)
Olfactory Epithelium, Metaplasia	1	(1.0)	8*	(1.3)	37**	(1.5)	49**	(2.2)
Respiratory Epithelium, Hyaline Degeneration	12	(1.0)	14	(1.0)	45**	(1.6)	3*	(1.7)
Respiratory Epithelium, Hyperplasia	0		26**	(1.8)	50**	(2.5)	50**	(3.5)
Respiratory Epithelium, Squamous Metaplasia	0		0		3	(1.0)	26**	(1.4)
Lateral Wall, Adenoma	0		1		0		0	
Respiratory Epithelium, Adenoma[c]	0		0		1		0	
Respiratory Epithelium, Carcinoma[d]	0		0		0		1	
Respiratory Epithelium, Squamous Cell Carcinoma[d]	0		0		0		3	
Adenoma, Carcinoma, or Squamous Cell Carcinoma (Combined)	0		1		1		4*	

* Significantly different ($P \leq 0.05$) from the chamber control group by the Poly-3 test
** $P \leq 0.01$
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Historical incidence for 2-year NTP inhalation studies with untreated chamber controls (mean ± standard deviation): 1/897 (0.1% ± 0.5%); range, 0%-2%
[d] Historical incidence: 0/897
[e] Historical incidence: 1/892 (0.1% ± 0.5%); range, 0%-2%

The mouse renal tumors are the result of an exacerbated pathology that does not occur in humans

There was an increase in the combined incidence of renal adenomas and carcinomas in male mice exposed to 32 ppm, together with associated pathological changes indicative of renal tubular injury (*i.e.*, increased severity grade of nephropathy and renal tubule degeneration), as follows:

Table 5: Furfuryl Alcohol ♂ mouse kidney tumor incidence

Dose (ppm)	0	2	8	32
Number	50	50	50	50
Survival	34	36	30	38
Terminal bwt % controls	-	101	99	98
Nephropathy	49	48	43	47
Average grade	1.2	1.4	1.5	1.8
Renal tubule, degeneration	0	0	1	48**
Renal tubule, hyperplasia [a]	4	8	3	5
Renal tubule adenoma [a]	0	0	0	3
Renal tubule carcinoma [a]	0	0	0	2
Combined [a]	0	0	0	5*

** p≤ 0.01; Average severity grade of lesion in (); 1=minimal, 2=mild, 3=moderate, 4=marked
[a] This is combined result from first evaluation plus a second step section evaluation

It should be noted that the statistical increase was only apparent after the NTP had conducted a second evaluation of the kidneys which involved examining an additional 8 sections. This practice was not followed for the control animals (neither concurrent nor historical) and it was only possible to attain statistical significance by combining the adenoma and carcinoma results from the first and second evaluations.

Nephropathy consisted of necrosis and regeneration of renal tubule epithelium and inflammation and fibrosis in the interstitium. Renal tubule degeneration was separately diagnosed and consisted of slightly distended tubules with lumens containing eosinophilic, finely granular material. Some degenerate tubules had one to a few enlarged epithelial cells with large, sometimes pleomorphic, nuclei.

Chronic progressive nephropathy (CPN) is an age-related disease of spontaneous origin commonly observed and well characterized in rats (Hard 2004). A spontaneous chronic nephropathy is also a common occurrence in mice as noted in the furfuryl alcohol inhalation study where 49/50 control males and 41/50 control females were diagnosed with nephropathy. Frazier and Seely (2013) state "*while the classical and most studied presentation of CPN occurs in the rat, a similar but pathologically distinct renal lesion also occurs in the mouse. The clinical relevance of CPN in the mouse is thought to mirror CPN in rats, including a relationship to increased tubular epithelial proliferation in the kidney in chronic studies*".

There is growing evidence and consensus that advanced CPN is a risk factor for development of a background incidence of atypical tubule hyperplasia and renal tubule tumors (RTT) where this association has been extensively studied in rats. The likely cause underlying this association with tubule neoplasia is the long-term increased tubule cell proliferation that occurs throughout CPN progression. As a variety of chemicals are able to exacerbate CPN, there is a potential for those exacerbating the severity up to and including end-stage kidney disease to cause a marginal increase in RTT and their precursor lesions (Hard, 2013). Extensive statistical analysis of NTP studies shows a strong correlation between high-grade CPN, especially end-stage CPN, and renal tumor development in rats (Travlos, 2011). Since the clinical implications of nephropathy in mice mirror CPN in rats as mentioned above (Frazier and Seely, 2013), the implication of exacerbated CPN and enhanced tubular epithelial proliferation for renal tumor development in rats is relevant for understanding tumor development in mice. Since there is no counterpart to rodent CPN in humans (Hard 2013), renal tumors in rodents resulting from chemical enhancement of this spontaneous rodent pathological syndrome are not considered as a relevant indication of human risk.

A postulated sequence of key events for renal tumor development related to exacerbation of CPN in rats is as follows (Hard 2013):

(i) exposure to chemical (usually at high concentrations);
(ii) metabolic activation (if necessary);
(iii) exacerbated CPN, including increased number of rats with end-stage renal disease;
(iv) increased tubule cell proliferation because more kidney is damaged due to CPN exacerbation;
(v) hyperplasia;
(vi) adenoma (infrequently carcinoma).

It is considered that this sequence of events is applicable to the renal adenomas/ carcinomas observed in the male mouse kidneys. In the mouse furfuryl alcohol study, there is an increased exacerbation of CPN, with the average grade rising from 1.2 in the controls to 1.8 in the 32 ppm group.

As the occurrence of adenomas and carcinomas is restricted to the 32 ppm group where there is evidence of renal tubular injury and increased severity of CPN, it is concluded that the low incidence of renal tubular tumors in male mice is secondary to the chronic tubular injury.

Interpretation of NTP Studies by Other Authoritative Bodies and Expert Groups

The NTP study authors interpreted the study results differently than the CARC, and concluded that there was ‘some’ evidence of carcinogenic activity of furfuryl alcohol in male F344/N rats, ‘equivocal’ evidence of carcinogenic activity in female F344/N rats, ‘some’ evidence of carcinogenic activity of furfuryl alcohol in male $B6C3F_1$ mice and ‘no’ evidence of carcinogenic activity in female $B6C3F_1$ mice exposed to 1, 8, or 32 ppm furfuryl alcohol.)

A similar interpretation was made by the German Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission), whose task, in accordance with the statutes of the Deutsche Forschungsgemeinschaft,[1] is to provide scientific policy advice. The MAK Commission proposes maximum workplace concentrations (MAK values) for volatile chemicals and dusts, biological tolerance values (BAT values), biologische Leitwerte (BLW), biological reference values for workplace substances (BAR) and analytical methods for substances in the air and biological material. Substances which are carcinogenic, germ cell mutagenic, sensitizing or absorbed percutaneously or which pose a risk during pregnancy are classified accordingly.

In 2008, the MAK Commission (2012a) conducted a comprehensive formal review and evaluation of the 1999 NTP studies. The Commission determined that the available information is insufficient to classify furfuryl alcohol as a likely human carcinogen and assigned a classification of '3B' in the carcinogenicity category. The MAK noted that the slightly but significantly increased kidney tumor incidences in male mice occurred simultaneously with renal tubular degeneration as a sign of a cytotoxic effect. The Commission further noted that the slightly increased incidence in nasal epithelial tumors in male rats appeared likely to have been caused by chronic irritation. Indeed, the MAK noted that a NOAEC (No Observed Adverse Effect Concentration) for toxicity could not be derived from the data since local nasal irritation occurred in both species even at the lowest concentration level.

Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures includes furfuryl alcohol and classifies the chemical as "Carc Cat 2, H351" which is defined as a "Suspected human carcinogen…not sufficiently convincing to place the substance in Category 1A or 1B."

The Joint FAO/WHO Expert Committee on Food Additives (JECFA), published a draft monograph (WHO, 2001) on its evaluation of a group of 15 furfuryl derivatives including the parent furfuryl alcohol and its structurally related analogs and derivatives. These flavoring agents were grouped on the basis of the criterion that all are hydrolyzed and/or metabolized to furoic acid or a substituted furoic acid. Based on the predicted metabolism of these substances and data on their toxicity (including review of the NTP carcinogenesis studies) the Committee concluded that consumption of furfuryl alcohol and the 14 related substances at the current levels of intake would not raise concern for safety.

[1] The DFG (Deutsche Forschungsgemeinschaft or German Research Foundation) is an important German research funding organization that supports research in science, engineering and the humanities. The DFG is a self-governed organization based in Bonn and is financed by the German states and federal government. Only Germany's leading research universities are members of the DFG.

Occupational Exposure to Furfuryl Alcohol

The majority of furfuryl alcohol (85-90% worldwide) is used in the production of furan resins for foundry sand binders in the metal casting industry. Furan is a generic term for binders containing furfuryl alcohol and either urea- or phenol-formaldehyde, or mixtures of both. Currently, furfuryl alcohol is used mainly in binders for the traditional furan 'no-bake' system and in smaller quantities in furan hot-box, warm-box, and gas-hardened processes (Chemical Economics Handbook, 2011).

The current permissible inhalation exposure limit for furfuryl alcohol designated by the Occupational Safety and Health Administration is 50 ppm (200 mg/m^3), determined as a time-weighted average for up to an 8-h work shift. The NIOSH recommended exposure limit is 10 ppm (40 mg/m^3) with a short-term exposure limit of 15 ppm (60 mg/m^3). An immediately dangerous to life and health value of 75 ppm was also set by NIOSH based on acute inhalation toxicity data in animals (NIOSH, 2015).

Epidemiological Studies

We are unaware of any epidemiological studies where exposure to furfuryl alcohol can be directly correlated with health effects because of the presence of other, more toxic, chemicals in the same studies. The following summary, which was excerpted from a MAK Commission report on furfuryl alcohol, provides information from several studies in which furfuryl alcohol was one of the target chemicals in the study (MAK Commission, 2012b).

> The odour threshold for furfuryl alcohol was determined in 13 test persons as 7–8 ml/m^3 (Jacobson *et al.* 1958).
>
> Of 17 foundry workers subjected to lung function tests, 9 were shown to have reduced FEV_1 values. The furfuryl alcohol concentrations measured ranged up to 50 ml/m^3, formaldehyde concentrations were about 4 ml/m^3. Because of the presence of other substances and the relatively high formaldehyde concentrations, the effects on lung function could not be ascribed to the furfuryl alcohol exposures (Low and Mitchell, 1985). In three test persons, oral administration of 0.6 to 1.0 g furfuryl alcohol as a 5 % aqueous solution resulted in an increase in respiration rate (Erdmann, 1902).
>
> Dermatitis and irritation of the respiratory passages was seen in bricklayers who came into contact with an acid-resistant cement containing furfuryl alcohol. The effects, however, could not be ascribed unambiguously to furfuryl alcohol (Mastromatteo, 1965).
>
> In a foundry in which a mixture of furfuryl alcohol, paraformaldehyde, phosphoric acid and sulfuric acid was poured onto hot sand, eye irritation with lacrimation developed after about 15 minutes in two persons exposed to a concentration of 15.8 ml/m^3 furfuryl alcohol; the formaldehyde concentration was 0.33 ml/m^3. At furfuryl alcohol

concentrations of 8.6 and 10.8 ml/m^3, the irritation did not develop. It is, however, not clear which of the substances caused the eye irritation seen at the highest furfuryl alcohol concentration (NIOSH, 1979). None the less, the American threshold limit value of 10 ml/m^3 is based on these findings (*Federal Register* 1989).

In an unpublished study, no evidence of eye irritation was seen in workers exposed during an 8-hour shift at a similar workplace to furfuryl alcohol concentrations up to 15 ml/m^3 (NIOSH, 1979).

Overall Conclusions:

We disagree with the description of the tumors as 'rare' and the CARC classification of furfuryl alcohol as "Likely to Be Carcinogenic to Humans" based on treatment-related nasal and kidney tumors reported in rodents.

In the mouse, occurrence of renal adenomas and carcinomas is restricted to only the 32 ppm (highest concentration) group along with associated rodent-specific and sex-specific pathological changes indicative of chronic progressive renal tubular injury. Since there is no counterpart to rodent chronic progressive nephropathy in humans, renal tumors in rodents resulting from chemical enhancement of this spontaneous rodent pathological syndrome are not considered as a relevant indication of human risk.

In the rat, changes in nasal pathology were observed at all dose levels but neoplastic changes were only observed in males at the highest dose; this dose was also associated with increased mortality and decreased bodyweight gain and is considered to have exceeded the maximum tolerated dose.

With the sole exception of the CARC recommendation, we did not find any scientific or government organization that has concluded that furfuryl alcohol is a likely human carcinogen after evaluating the NTP studies. Furfuryl alcohol has been commercially available since 1953, and we could not find any epidemiological evidence that chronic exposure to furfuryl alcohol causes cancer.

We wish to thank OEHHA in advance for consideration of the issues presented above in justification of our position that furfuryl alcohol should <u>not</u> be listed as a carcinogen under Proposition 65. We would sincerely appreciate the opportunity to meet with OEHHA for further discussion of these issues.

Respectfully submitted on behalf of all Respondents listed on the following page,

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ATTACHMENT A:

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TOXICOLOGIC PATHOLOGY

NONCLINICAL SAFETY ASSESSMENT

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12 Urinary System

Kendall S. Frazier and John Curtis Seely

CONTENTS

12.1 Kidney 422
12.1.1 Introduction 422
12.1.1.1 Functional Anatomy 423
12.1.1.2 Embryology 426
12.1.1.3 Ancillary Tests of Renal Function or Damage 426
12.1.2 Glomerular Changes 429
12.1.2.1 Glomerulonephritis 432
12.1.2.2 Mesangioproliferative Glomerulopathy 434
12.1.2.3 Hyperplasia, Mesangial 435
12.1.2.4 Glomerulosclerosis 436
12.1.2.5 Hyaline Glomerulopathy 437
12.1.2.6 Mesangiolysis 437
12.1.2.7 Amyloidosis 438
12.1.2.8 Glomerular Atrophy 439
12.1.2.9 Bowman's Space Enlargement 439
12.1.2.10 Metaplasia and Hyperplasia of Bowman's Capsule 439
12.1.3 Tubular Changes 440
12.1.3.1 Tubular Degeneration and Tubular Basophilia 440
12.1.3.2 Vacuolation 440
12.1.3.3 Renal Phospholipidosis 441
12.1.3.4 Diabetic Nephropathy and Tubular Glycogenosis 442
12.1.3.5 Tubular Dilation and Cystic Tubules 443
12.1.3.6 Casts 444
12.1.3.7 Necrosis 444
12.1.3.8 Infarction 446
12.1.3.9 Tubular Atrophy 447
12.1.3.10 Tubular Regeneration 447
12.1.3.11 Karyomegaly 448
12.1.3.12 Tubular Hypertrophy 448
12.1.3.13 Chronic Progressive Nephropathy 449
12.1.3.14 Hyaline Droplets and α-$_{2U}$-Globulin Nephropathy 450
12.1.3.15 Crystalluria, Obstructive Nephropathy, and Retrograde Nephropathy 451
12.1.4 Papillary Changes 453
12.1.4.1 Papillary Necrosis 453
12.1.4.2 Pyelonephritis 454
12.1.5 Interstitial and Vascular Changes 455
12.1.5.1 Interstitial Inflammation and Interstitial Nephritis 455
12.1.5.2 Interstitial Fibrosis 456
12.1.5.3 Periarteritis and Vasculitis 457

While the etiology of CPN is still largely controversial, the incidence of the condition is influenced by various physiological factors such as caloric intake, protein content of diet, and male hormones. The total amount of ingested food over a long period seems to be a major determinant of the severity of spontaneous damage (Keenan et al. 2000). Castration is protective in the male but has no effect in females, and chronic androgen administration will accelerate damage in females, suggesting an important androgen influence (Baylis 1994). Neither vascular lesions nor immune complexes are associated with the pathogenesis of the lesion. While there are concurrent glomerular lesions associated with the tubular changes and glomerular hyperfiltration is involved to some degree in the development of glomerulosclerosis, the historical hypothesis that the pathogenesis of CPN-mediated tubular injury is also related to glomerular hyperfiltration is controversial. There is no established causal relationship between glomerular capillary pressure and degree of glomerular or tubular damage in studies of CPN (Baylis 1994). Albuminuria associated with advanced CPN may be due more to failure of postglomerular cellular processing of albumin by tubules rather than to changes in glomerular permeability, since albumin is more freely filtered than once thought (Russo et al. 2002). Rather than a primary glomerular lesion, the primary pathogenesis of CPN is now thought to involve changes in the basement membranes throughout the kidney. Alterations in the amino acid composition, hydroxylation, and glycosylation of basal lamina surrounding tubules are noted in some of the earliest lesions of CPN (Abrass 2000). The thickened basal lamina likely disturbs epithelial attachments to the basement membrane leading to cytoskeletal and functional alterations provoking cytoplasmic basophilia in the proximal tubules and altered filtration in the glomeruli. Changes in tinctorial characteristics of the PCTs are an initial and nonspecific hallmark of the nephron's response to injury, so basophilia is one of the earliest microscopic lesions that can be noted by the pathologist, but thickened basement membranes are visualized soon thereafter.

While the classical and most studied presentation of CPN occurs in the rat, a similar but pathologically distinct renal lesion also occurs in the mouse. This condition is not as well characterized as CPN in the rat, and while a similar constellation of changes occurs progressively over time, it has unique features and appears to have a separate pathogenesis. The incidence in mice is significantly less than in rat, and the age of onset is later, with the earliest evidence of lesions usually appearing only after 4 months of age. Tubular basophilia and nuclear crowding are noted, but thickening of the basal lamina is harder to appreciate except in the more chronic cases. Hyaline casts increase in number over time. Mononuclear inflammatory infiltrates are more prominent in mice than in rats with CPN and tend to expand the interstitium. Dilation of the glomerular space is often present, but glomerulosclerosis occurs only in the later stages of the disease. The clinical relevance of CPN in the mouse is thought to mirror CPN in rats, including a relationship to increased tubular epithelial proliferation in the kidney in chronic studies.

12.1.3.14 Hyaline Droplets and α-$_{2U}$-Globulin Nephropathy

Hyaline droplets are frequently found in the cytoplasm of proximal tubules in male rats, generally localized to the S2 segment, and are characterized by variably sized, refractile, brightly eosinophilic ovoid droplets consisting of lysosomal accumulations of α-$_{2U}$-globulin. There are a large number of agents (d-limonene, hydrocarbon or petroleum products, decalin) that will result in α-$_{2u}$-globulin nephropathy, characterized by an increase in droplet number and distribution, and/or altered pattern of droplet distribution (Dill et al. 2003). Although the hyaline droplets are most often not associated with any other visible evidence of tubular injury, in more severe cases, there may be occasional single cell necrosis, sloughed luminal epithelial cells, or increased mitoses of tubules, and the distribution may include all segments of the proximal tubules. In chronic cases, granular casts may form at the junction of the inner and outer medullary stripes, and linear medullary and papillary mineralization has been described (Hard et al. 1993). In carcinogenicity studies using compounds that induce α-$_{2U}$-globulin nephropathy, tubular hyperplasia and increased incidence of renal tumors have also been described (Hard et al. 1999). The change is frequently accompanied by exacerbation of the incidence or severity of spontaneous CPN (Dill et al. 2003). While test article administration

ATTACHMENT B:

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[98-00-0]

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TLV	-
STEL	-
Absorption through the skin (1998)	H
Sensitization	-
Carcinogenicity (2007)	Category 3 B
Teratogenic effect	-
Germ cell mutagens effect	-
BAT value	-

1. General toxicological profile

In a two years inhalation study in F344 rats , Furfuryl alcohol shows a slightly increased incidence of nasal tumors and a questionable increased incidence of renal tumors..
Renal tumors are also found in male B6C3F1 mice. From the lowest tested concentration of 2 ml / m3 in the 2 -year trial, signs of local irritation occurs at the nose of rats and mice.
Furfuryl alcohol is a skin and eye irritant in animal experiments.
In vitro, the substance is questionable clastogenic in mammalian cells but not mutagenic in the Salmonella mutagenicity test .
In vivo genotoxicity in mice are negative.
For workers there is an average exposure of 7 mg furfuryl alcohol / m3 (1.75 ml / m3)
and peak exposures up to 40 mg / m3 (10 ml / m3) by which restriction of lung function parameters and irritation of the respiratory tract and the eye occurs.
Furfuryl alcohol is acutely toxic by the oral and inhalation exposure in the rat and after dermal application in rabbits.
For acute toxicity there are marked differences between species .
Studies on allergenic effects are absent, as special studies on Reproductive toxicity.

2. Mode of Action

Furfuryl alcohol is metabolized via the oxidation product furfural, therefore,
the reader is referred to the assessment " 2- Furylmethanal (furfural) " from 1994 .
After oral administration of high doses of furfural to rats Cholangiocarcinomas
or their precursors on are formed, which is initiated on the mouse skin and leads
genotoxic effects in vitro. Therefore, it was classified as a carcinogenicity category 3 B.

The tumors of the nose of rats be explained by the irritant effect of furfuryl alcohol
A local genotoxicity , mediated by furfural could , in the development of tumors play a role.
In vivo, the substance is not genotoxic.
However, as it was only a questionable positive in the in vitro genotoxicity tests with furfuryl during alcohol metabolic activation in two chromosome aberration tests and the Salmonella mutagenicity tests were negative , the genotoxic potential , if at all, should be only weak and for the range of the nasal tumors play a minor role.
In the case of renal tumors is unclear whether this due to a reactive genotoxic
Metabolite (furfural) or whether it is caused by a cytotoxic effect .

3. Toxicokinetics and Metabolism

3.1. Absorption , distribution, excretion

Up to 72 hours after oral administration of 0.275 -27.5 mg radiolabelled furfuryl alcohol/kg to male F344 rats, approximately 88 % of the administered dose were found in urine as metabolites , 4 % of the dose was excreted in the faeces.
14CO2 was not detected in the breath . The highest concentrations of radioactivity were found in liver and kidney, the lowest in brain (Nomeir et al. 1992).

3.2. Metabolism

The excreted 2- furoic acid of the exposed worker was linear with the furfuryl alcohol concentration and the correlated exposure time (Pfäffli et al . 1985).
After oral administration of 0.275 mg -100 furfuryl alcohol / kg to male F344 rats following metabolism was detected: furfuryl alcohol is to first oxidezed to furfural oxized. Furfural is either further oxidized to 2- furoic acid (1 % -6) and then conjugated with glycine to the main metabolite 2- Furoylglycin (73 -80 %) or , presumably with acetic acid to 2- furanacrylic acid (3 % -8) and converted to 2-furanacryloylglycin (Figure 1 ; Irwin et al , 1985; . Nomeir et al., 1992) .
In a 2-year inhalation study with rats also 2- Furoylglycin was found as major metabolite in the urine next to 2 Furanacryloylglycin (about 5% of 2- Furoylglycin-Amount) (NTP 1999).

4. Human experience

In a foundry the lung function of 28 workers using dynamic spirometry and an additional 11 exposed workers using static Spirometry investigated.
The workers were exposed for an average of 15 years. The time-weighted average exposure to furfuryl alcohol was on average 7 mg / m3 (1.75 ml / m3) up to 15 mg / m3 (3.75 ml / m3),by which the short-term peak exposure peaks 40 mg / m3 (10 ml / m3) is exceeded . In addition, there was an exposure to < 2 mg respirable dust / m3 and 0.4 mg formaldehyde / m3 (approximately 0.3 ml / m3).
Among the 28 workers , the frequency was to respiratory problems: (Cough 6/28 , 8/28 nose , sore 11/28 , control subjects : 1/27 , 3/27 , 0/27) and the Eyes problems increased (3/28 , control subjects : 1/27) and the vital capacity after the workday (- 0.18 l; $p < 0.05$) lower. The 11 other exposed accounted after the work day a decrease in total lung capacity. The authors concluded the observations as an acute lung function restriction with an unclear mechanism. A chronic impairment of lung function was not found , because the lung function values were similar before the workday to that of the control subjects (Åhman et al . 1991).

5. Animal experiments and in vitro studies

5.1. Acute toxicity

For this endpoint, there is no new data.

5.2. Sub-acute , subchronic and chronic toxicity

5.2.1. Inhalation toxicity

In an unpublished study from 1952 were 15 rats and 8 mices (k.w a) exposed for 6 hours per day, 5 days per week during 3 weeks to 19 ml / m3 . Compared to control animals , the body weight gain was not affected. There was diffuse congestion throughout the respiratory tract, but no other macroscopic findings in other organs (NTP 1999).
In a 16-day study, each 5 male and female F344 rats per dose group was exposed to 0, 16, 31, 63, 125 or 250 ml furfuryl alcohol / m3 6 hours /Day, 5 days / week, and the whole body was exposed. The body weight gain of the male animals as of 31ml/m³, the female as of 125ml/ m3 max decreases from 15%.

As of 63 ml / m3 dyspnea, hypoactivity, nasal and ocular discharge. At 250 ml / m3 all animals died, at125 ml / m3 one male animal died. All exposed animals showed a concentration-dependent acute inflammation of the nasal cavity or Necrosis, regeneration and metaplasia in the respiratory epithelium andNecrosis and degeneration of the olfactory epithelium. A NOAEC could can not be determined (NTP 1999).

In a 16-day study, each 5 male and female B6C3F1 mice were per concentration group to 0, 16, 31, 63, 125 or 250 ml furfuryl alcohol / m3 6 hours /Day, 5 days / week, whole body exposed. The body weight gain of the maleand females was reduced for the group exposed to 63ml/ m3 with maximum 12%. At 250 ml / m3 all animals were killed, at 125 ml / m3 one female animal died. All exposedAnimals, except for one male at 16 ml / m3, showed concentration-dependent histologica , changes in the nasal respiratory or olfactory epithelium (cf.analogous experiment in rats). These changes were at 16 ml / m3 were described as minimal. A NOAEC could not be determined (NTP 1999).

In a 14-week study of 10 male and female F344 rats were exposedto 0, 2, 4, 8, 16 or 32 ml furfuryl alcohol / m3 6 hours / day, 5 days / week (full body exposed). Concentration-dependent and statistically significant was noted in males and females from 2 ml / m3 squamous metaplasia of the transitional epithelium. At 4 ml / m3, the incidence of degeneration of the olfactory was Epithelium increased, at 8 ml / m3 changes in the respiratory epithelium to squamous and Becherzellhyperplasian were noted. As of 16 ml / m3 occurred in upper respiratory tract. Hypertrophy of the respiratory epithelium and surface exudate of the olfactory epithelium and cellular infiltrates in the lamina propria on. At 32 ml / m3, the incidence of metaplasia in the olfactory epithelium of the female Animals and the number of Spermatids and the number of Spermatidenheads increased. The mobility and the concentration of spermatozoa was not altered. There were no significant differences with respect to the vaginal cytology. A NOAEC could not be determined (NTP 1999).

In a 14-week study of 10 male and female B6C3F1 mice were exposed to 0, 2, 4, 8, 16 or 32 ml furfuryl alcohol / m3 6 hours / day, 5 days / week,(Full body exposed) . Concentration-dependent and statistically significant, there was at 2 ml / m3 degeneration and metaplasia in the olfactory epithelium of male. Animals and hyaline droplets in the respiratory epithelium of the female animals. At 4 ml / m3 chronic inflammation of the olfactory epithelium in male Animals and degeneration of the olfactory epithelium of the female animals were reported. At 8 ml / m3 were the incidences of squamous metaplasia of the submucosal glands the cubic epithelium in male and metaplasia and chronic inflammation increases in female animals. As of 16 ml / m3 there was hyaline droplets in the respiratory epithelium of male and squamous metaplasia of the submucosal Glandular epithelium of the cubic of the female animals. There were no significant cant differences with respect to sperm motility and vaginal cytology. A NOAEC could not be determined (NTP 1999).

A 2-year study was conducted in F344 rats. Each 50 male and female Animals were exposed to 0, 2, 8, or 32 ml furfuryl alcohol / m3 6 hours / day, 5 Days / week, (whole body exposed). The survival and body weight of the males were decreased at 32 ml / m3. The clinical findings showed no deviations from the norm. At 2 ml / m3 were irritation to the nasal mucosa on the incidence of which increased concentration dependent. The hyperplasia of the lateral nasal mucosa (respiratory or transitional epithelium) proved to be the most sensitive endpoint (the concentration-response relationship is so steep that can be calculated no meaningful benchmark concentration). The severity nephropathy increased concentration dependent. In male animals, the Incidence of mineralization in the kidney at 32 ml / m3

significantly increased. the increased incidence of parathyroid hyperplasia and fibrous osteodystrophy were interpreted by the authors as a sign of kidney failure as a result of furfuryl alcohol by reinforced nephropathy. A NOAEC was not obtained. The tumor incidences are described in Section 5.7.2 (Table 1; NTP 1999). the changes indicates a furfuryl alcohol-induced renal injury, with effect on the hormone-dependent bone metabolism.

In a 2-year study of 50 male and female B6C3F1 mice were compared 0, 2, 8, or 32 ml furfuryl alcohol / m3 6 hours / day, 5 days / week full body exposed. The survival and body weight of the male animals and the survival time of the females were normal. The body weight of the exposed female mice was lower than that of the control animals. Female mice exhibited at 32 ml / m3 focal corneal haze. As of 2 ml / m3 irritation to the nasal mucosa wh increased with increasing concentration dependent. At 32 ml / m3 the renal tubule degenration in male mice. The severity of Nephropathy is concentration dependent. A NOAEC was not obtained.

The tumor incidences are described in Section 5.7.2 (Table 1; NTP 1999).

Table. 1. Effects of furfuryl alcohol after 2 years of inhalational administration to rats and Mice (NTP 1999)

Concentration [ml/m3]	0	2	8	32
F344-Rats				
Nose				
– suppurative Inflammation				
_male	3a)/50b) (1,0)	6/50 (1,5)	17/50** (1,7)	44/50** (2,1)
_female	4/49 (2,3)	1/50 (2,0)	5/48 (1,4)	23/49** (1,7)
Bowman's glands				
– Hyperplasia				
_male	0/50	0/50	22/50** (1,0)	49/50** (2,3)
_female	0/49	0/50	24/48** (1,0)	46/49** (2,2)
Lateral wall				
– Hyperplasia				
_ male	1/50 (1,0)	49/50** (1,5)	50/50** (2,4)	50/50** (3,5)
_female	0/49	39/50** (1,3)	48/48** (2,1)	49/49** (3,5)
– Epithelmetaplasia				
_ male	1/50 (1,0)	1/50 (1,0)	8/50* (1,1)	33/50** (1,3)
_ female	0/49	1/50 (1,0)	0/48	24/49** (1,0)
Olfactory Epithelium				
– Atrophy				
_ male	1/50 (1,0)	12/50** (1,1)	47/50** (1,8)	50/50** (2,4)
_ female	0/49	6/50* (1,3)	44/48** (1,7)	49/49** (2,3)
– Fibrose				
_male	0/50	1/50 (1,0)	26/50** (1,0)	40/50** (2,0)
_female	0/49	0/50	16/48** (1,3)	31/49** (1,7)
– Hyperplasia				
_male	0/50	1/50 (1,0)	42/50** (1,0)	40/50** (1,8)
_female	0/49	0/50	31/48** (1,2)	41/49** (1,5)
– Metaplasia				
_ male	1/50 (1,0)	8/50* (1,3)	37/50** (1,5)	49/50** (2,2)
_ female	0/49	5/50* (1,2)	37/48** (1,5)	48/49** (2,2)
Respiratory Epithelium				
– Hyperplasia				

_	0/50	26/50** (1,8)	50/50** (2,5)	50/50** (3,5)
_	0/49	18/50** (1,4)	40/48** (2,1)	49/49** (3,2)
– Epithelium metaplasia				
_ male	0/50	0/50	3/50 (1,0)	26/50** (1,4)
_ female	0/49	0/50	2/48 (1,0)	10/49** (1,2)
Kidney				
– Mineralisation				
_male	2/50 (3,0)	2/50 (3,0)	2/50 (4,0)	28/50** (3,3)
_female	0/50	1/49 (2,0)	0/49	0/50
– Nephropathy				
_ male	50/50 (2,9)	49/50 (2,9)	50/50 (3,1)	50/50 (3,7)**
_female	47/50 (1,9)	45/49 (1,9)	47/49 (1,9)	47/50 (2,4)**
Parathyroid glands				
– Hyperplasia				
_ male	9/49 (2,1)	5/45 (3,0)	12/50 (2,4)	39/49** (3,6)
Bone				
– fibrous Osteodystrophy				
_male	2/50 (2,0)	5/50 (2,2)	6/50 (2,5)	34/50** (3,2)
B6C3F1-Mäuse				
Nose				
– suppurative Inflammation				
_ male	7/50 (1,4)	11/49 (1,2)	27/49** (1,3)	28/50** (1,7)
_female	5/50 (1,2)	12/48** (1,1)	25/49** (1,5)	42/50** (2,0)

Tab. 1. Fortsetzung

Concentration [ml/m3]	0	2	8	32
Bowman's glands				
– Hyperplasia				
_ male	0/50	10/49** (1,2)	48/49** (1,3)	46/50** (1,7)
_female	0/50	33/48** (1,1)	46/49** (2,8)	47/50** (3,1)
– Epithelium metaplasia				
_male	0/50	6/49* (1,0)	35/49** (1,1)	47/50** (1,5)
_female	1/50	1/48 (1,0)	34/49** (1,1)	46/50** (1,5)
Lateral wall				
– Epithelium metaplasia				
_male	0/50 (1,0)	9/49** (1,0)	10/49* (1,7)	20/50** (1,5)
_female	3/50 (1,0)	14/48** (1,4)	16/49** (1,4)	36/50** (1,9)
Olfactory Epithelium				
– Atrophy				
_male	1/50 (1,0)	12/49** (1,1)	47/49** (1,8)	50/50** (2,4)
_female	0/50	6/48* (1,3)	44/49** (1,7)	49/50** (2,3)
– hyaline Degeneration				
_male	2/50 (1,5)	3/49 (1,7)	21/49** (1,3)	39/50** (2,0)
_ female	7/50 (1,3)	14/48 (1,4)	28/49** (1,8)	45/50** (2,2)
– Metaplasia				
_ male	0/50	12/49** (1,0)	49/49** (1,0)	50/50** (1,8)
_ female	0/50	31/48** (1,2)	49/49** (3,0)	49/50** (3,6)
Respiratory Epithelium				
– hyaline Degeneration				
_ male	5/50 (1,0)	18/49** (1,1)	42/49** (1,3)	45/50** (1,2)
_ female	19/50 (1,4)	44/48** (1,5)	49/49** (1,3)	48/50** (1,4)
– Epithelium metaplasia				
_male	0/50	2/49 (1,0)	10/49** (1,1)	20/50** (1,4)
_female	1/50 (1,0)	9/48** (1,8)	21/49** (1,7)	39/50** (1,9)
– Necrosis				
_male	1/50 (2,0)	0/49	0/49	1/50 (1,0)
_female	0/50	0/48	2/49 (2,5)	3/50 (1,3)

– Regeneration				
_male	0/50	1/49 (1,0)	13/49** (1,0)	21/50** (1,0)
_female	0/50	0/48	9/49** (1,0)	13/50** (1,2)
Kidney				
– Nephropathy				
_male	49/50 (1,2)	48/49 (1,4)	43/49 (1,5)	47/50 (1,8)
_female	41/50 (1,0)	35/48 (1,1)	40/49 (1,2)	39/49 (1,0)
Kidneytubuli				
– Degeneration				
_ male	0/50	0/49	1/49 (1,0)	48/50** (1,0)

a) Animal concerned , average severity (1 -4, minimal to clear) hyperplasia in the paranthesis
b) examined Animal
* p<0,05, **p<0,01 against control group by the Poly-3 test (incidences) or Mann-Whitney U test (severities)

5.2.2. Ingestion

In an unpublished study from 1952 moderate degeneration of hepatocytes and Epithelial cells in the renal cortex occurred after doses of 75, 150 rat tubular or 300 mg / kg bw. In mice ingesting 300 or 600 mg / kg body weight, these findings were more pronounced and included necrosis and vacuolation in the cytoplasm (k.w. A .; NTP 1999).

Male and female F344 rats and B6C3F1 mice were during 13 weeks long exposed to 0, 38, 75, 150 or 300 mg furfuryl alcohol / kg body weight by gavage, mice additionally exposed to 600 mg / kg body weight. As of 50 mg / kg body weight, all rats died , as of 300 mg / kg some of the mice died. The absolute liver and kidney weights of the rats increased at 75 mg / kg bw . The body weights of the mice decreased at 600 mg / kg body weight with 15%. Other body weight differences were not reported neither in rats nor in the other groups of mice (unpublished data, kw A .; NTP 1999).

5.3. Effects on skin and mucous membranes

Furfuryl alcohol is a skin irritant in rabbits (ECB 2000) and irritating to eyes (Justification 1992, ECB 2000). In rats and mice, the substance is irritating to the nasal mucosa (see. Section 5.2.1 .; NTP 1999).

5.4. Allergens effect

Studies on this endpoint are still missing.

5.5. Reproductive toxicity

Studies on this endpoint are still missing.

After 14 weeks of inhalation exposure of rats at 32 ml furfuryl alcohol / m3 the spermatid count and the spermatid heads per testis increased. The sperl motility and the spermatozoal concentration were not changed. There were no significant differences in vaginal cytology on (see section 5.2.1;. NTP 1999).

In an analogous study in mice, there were no significant changes in the Spermatid count, sperm motility and vaginal cytology at exposure against up to 32 ml furfuryl alcohol / m3 (see Section 5.2.1;. NTP 1999).

5.6. Genotoxicity

In the context of the NTP, various genotoxicity tests were carried out in the are presented below (NTP 1999). More genotoxicity test (Salmonella Mutagenicity, SCE negative in vitro; Chromosomal aberrations in vitro positive, Drosophila negative) are described in the Explanatory Memorandum, of 1992.

5.6.1. In vitro

SCE in CHO cells were made in the absence, but not with the addition of S9 mix Rat liver by 16 -500 mg furfuryl alcohol / ml caused. Chromosome aberrations CHO cells were up to 500 mg furfuryl alcohol / ml without S9 mix does not occur. With the addition of S9 mix was in the first Test at 500 and 1000 mg furfuryl alcohol / ml of an increase in the aberrations found in the second (up to 1600 mg / ml) not, so the result was assessed as questionable (NTP 1999). in the described in the Explanatory Memorandum (1992) positive in vitro test for chromosomal aberrations with CHO cells increased concentrations of 2.5 mM (250 mg Furfuryl alcohol / ml) with metabolic activation, the number of chromatid breaks and - austauschen. Without metabolic activation was the effect of furfuryl alcohol weaker (stitch et al. 1981). NTP (1999) criticized the form shown in this publication, which made the results difficult to assess.

5.6.2. In vivo

Furfuryl alcohol induced intra peritoneal injection to the MTD (150 to 300 mg / kg KG) in mice no SCE nor chromosomal aberrations or micronuclei in the bone marrow. The SCE analysis followed after 23 or 42 hours post analyses, the Scoring aberrations 17 or 36 hours post analysis The investigation on micronuclei carried out 24 hours after the last of 3 applications at 24 Hours, the PCE / NCE ratio was, however, not dose-dependent changes (Abbott et al, 1991;. NTP 1999)

In a chromosomal aberration test Swiss albino mice received 0.5 ml of a furfuryl alcohol aqueous solutions with a content of 0.1; 0.2 or 0.4% per Gavage (approximately 0.5, 1 or 2 mg / animal, 25, 50 and 100 mg / kg bw adopted at a Body weight of 20 g). A determination of the MTD was not made. the Application was made either once or once a day for 5 days. Each 2 animals per dose were examined 6, 12, 18, 24, 36, 48 or 72 hours after the last application. There was a dose-dependent reduction of the mitotic index by furfuryl alcohol, only in the group at the highest dose with a 15% significance of the 5% level. In both treatment regimens, the incidence of chromosome aberrations was 0.4% 12 to 36 hours p. a. reported as significantly increased (Sujatha and Subramanyam 1994). In this study, only 2 Animals per dose group and time interval were used. That is not the according the OECD guideline 475 and allowed no acceptable statistical analysis. Other weaknesses in the work consist in the lack of definition of aberration types, such as the distinction between gaps and breaks, between "centric fusions" and "centromeric associations "between fragments and terminal deletions. The representation the data in the tables is sufficient, since only absolute Aberrations numbers are specified without making reference to the evaluated cell number. Overall, this work so inadequate that it can not be used as evidence of clastogenic effect of furfuryl alcohol.

5.7. Carcinogenicity

5.7.1. Short-term tests

In the SHE-test furfuryl alcohol in the cytotoxic concentration range not cell-transforming (Kerckaert et al. 1996).

5.7.2. Long-term studies

As described under 5.2.1 during the 2-year study tested at concentrations of 0, 2, 8, or 32 ml furfuryl alcohol / m3, the animals were examined for the presence of tumors at the end of the exposure period.

(Table 2). Because of a questionable increased incidence of tumors compared to the standard during the examination of the kidneys , an extended analysis was performed with 8 additional sections per kidney. In F344 rats it led to the formation of adenomas and carcinomas of the nasal mucosa, their frequency in the highest concentration group in males was significantly increased compared to the control group. In male rats only after evaluation Kidney sections a marginal increase of kidney tumors was reported but was considered by the authors as not substance-related. In the female animals, the kidney adenoma incidence was marginally increased and the concentration-dependent, but was reported as not significant. According to the authors there is a uncertain connection in the administration of the substance despite the rare occurrence of renal tumors in female rats, as with a 16-fold increase in exposure concentration the number of kidney adenoma only marginally increased, although the kidney tubulus hyperplasia increased significantly. In mice, it came in the nose not to tumors, but at the lowest concentration to precursors such as metaplasias in the respiratory and olfactory epithelium. In male mice, in the highest concentration group the renal tumor incidence was increased and was also higher than the highest range of the historical controls (inhalation studies until 1996 from the contract laboratory NTP for this study). This applies to both adenomas, as well as carcinomas. The tumors occurred only at the highest concentration group with simultaneous degeneration of the renal tubules which can been seen as evidence of renal toxicity. The evaluation of the NTP was "some evidence" for male rats, due to the nasal tumors, "equivocal evidence" for female rats due to kidney tumors, "Some evidence" for male mice, and because of renal tumors "No evidence" for female mice. The Commission shall evaluate the nasal tumors of rats and renal tumors of mice as substance-related and relevant to the humans.

Tab. 2. incidences of tumors and their precursors in the 2 -year study in F344 rats and B6C3F1 mice (NTP 1999

Author: NTP 1999
Substance Furfurylalcohol (purity>98%)
Species: Rat, F344/N, je 50 _,
Application: Inhalation
Concentration: 0, 2, 8, 32 ml/m3
Duration: 2 years, 5 days/week, 6 Hours/day
Toxicity : from 2 ml / m3 local irritant effect , survival time and the KG _ Animal the highest Concentration group
Tumors:

Concentration [ml/m3]	0	2	8	32
Nose				
Lateral wall				
– Adenoma				
_male	0a)/50b)	1/50 (2%)	0/50	0/50
_ female	0/49	0/50	1/48 (2%)	0/49

Respiratory Epithelium				
– Adenoma				
_male[c)]	0/50	0/50	1/50 (2%)	0/50
_female[d)]	0/49	0/50	0/48	1/49 (2%)
– Carcinomas				
_[e)] male	0/50	0/50	0/50	1/50 (2%)
– Squamous Cell Carcinoma				
_[e)] male	0/50	0/50	0/50	3/50 (6%)
Sum Nose tumors				
_ male	0/50	1/50 (2%)	1/50 (2%)	4/50* (8%)
_ female	0/49	0/50	1/48 (2%)	1/49 (2%)
Kidney				
– Kidneyntubulushyperplasia				
_[f)] male	10/50 (2,4)	7/50 (2,3)	7/50 (2,3)	25/50** (2,8)
_[f)] female	2/50 (1,0)	1/49 (2,0)	3/49 (2,0)	11/50* (2,0)
– Adenoma				
_[g, h)] male	1/50 (2%)	1/50 (2%)	2/50 (4%)	0/50
_[g, i)] female	0/50	0/49	0/49	2/50 (4%)
_[f)] male	2/50 (4%)	2/50 (4%)	3/50 (6%)	4/50 (8%)
_[f)] female	0/50	0/49	2/49 (4%)	2/50 (4%)
– Carcinomas				
_[g, j)]male	0/50	1/49 (2%)	0/49	0/50
_[f)] female	0/50	1/49 (2%)	0/49	0/50
Sum Kidneyntumors				
_[g)] male	1/50 (2%)	1/50 (2%)	2/50 (4%)	0/50
_[g, k)]female	0/50	1/49 (2%)	0/49	2/50 (4%)
_[f)] male	2/50 (4%)	2/50 (4%)	3/50 (6%)	4/50 (8%)
_[f)] female	0/50	1/49 (2%)	2/49 (4%)	2/50 (4%)

Author:	NTP 1999
Substance:	Furfurylalcohol (Purity>98%)
Species:	Mice, B6C3F1, je 50 _, _
Appliction:	Inhalation
Concentration:	0, 2, 8, 32 ml/m3
Duration:	2 years, 5 days/week, 6 Hours/day
Toxicity:	from 2 ml/m3 local irritation
Tumors:	

Concentration [ml/m3]	0	2	8	32
Kidney				
– Kidneyntubulushyperplasia[f)]				
_male	4/50 (1,5)	8/49 (1,0)	3/49 (1,0)	5/50 (2,0)
– Adenoma				
_[g, l)] male	0/50	0/49	0/49	2/50 (4%)
_[f)]female	0/50	0/49	0/49	3/50 (6%)
– Carcinomas				
_[g, m)] male	0/50	0/49	0/49	2/50 (4%)
_[f)] female	0/50	0/49	0/49	2/50 (4%)
Sum Kidneyntumors				
_[g, n)] male	0/50	0/49	0/49	4/50 (8%)
_[f)] female	0/50	0/49	0/49	5/50* (10%)

a) Animal concerned , average severity (1 -4, minimal to clear) hyperplasia in the paranthesis
b) examined Animal

c) historical control incidence: 1/897 (0,1%±0,5%), Range: 0 –2%
d) historical control incidence: 1/892 (0,1%±0,5%), Range: 0 –2%
e) historical control incidence: 0/897
f) Sum of the incidence from standard evaluation and additional 8 slides per Kidney
g) Standard evaluation
h) historical control incidence: 9/902 (1,0%±1,2%), Range: 0 –4%
i) historical control incidence: 1/898 (0,1%±0,5%), Range: 0 –2%
j) historical control incidence: 4/898 (0,5%±0,9%), Range: 0 –2%
k) historical control incidence: 5/898 (0,6%±0,9%), Range: 0 –2%
l) historical control incidence: 3/1093 (0,3%±0,6%), Range: 0 –2%
m) historical control incidence: 1/1093 (0,1%±0,4%), Range: 0 –2%
n) historical control incidence: 4/1093 (0,4%±1,0%), Range: 0 –4%
* p<0,05, ** p<0,01 against control group by the Poly-3 test (incidences) or Mann-Whitney U test (severities),

6. Evaluation

Furfuryl alcohol induced in male rats by inhalation slight, but significantly increased incidences of tumours in the nasal epithelium and in female rats not significantly increased incidences of kidney tumours. The kidney tumours occurred in female animals at the highest concentration on, which is also showed an increased nephropathy. In male mice, the kidney tumour incidences were light, but significantly increased with simultaneous renal tubular degeneration as signs of a cytotoxic effect.

The nasal tumours could be formed by genotoxic metabolites of furfural or because the only questionable positive results in in-vitro chromosome aberration test and the negative results in vivo with furfuryl alcohol is likely to be caused by irritation. Since the mechanism of formation of kidney tumours is unclear, furfuryl alcohol is classified as carcinogen category 3 B.

A MAK value can not be derived from the data, since no NOAEC was obtained. From the lowest tested concentration of 2 ml / m3 signs of local irritation occurred at the nose of rats and mice in the 2-year trial.

Workplace experience indicate that as of 1.75 ml / m3 with peak concentrations over 10 ml / m3 there is irritation of the respiratory organs and the eyes. The previous MAK value of 10 ml / m3 is too high and suspended. An occupational exposure limit must be well below 2 ml / m3.

There are no new data for skin sensitization. The substance therefore does not remain with "Sa" or "Sh" marks.

For skin absorption there are no new data. The substance is due to its low dermal LD50 in rabbits already marked with "H" (Supplement 1998). Thismarker is maintained.

The valid in vivo genotoxicity tests were negative, a local genotoxic potential due to an earlier positive in vitro study of chromosomal aberrations are not excluded. The available data lead not to a classification as a germ cell mutagen.

Studies on developmental toxicity are still not available.

Since no MAK value can be derived, it is not allocated to pregnancy group.

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Closed on 22.06.2006

ATTACHMENT C:

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Furfuryl alcohol

Classification/MAK value:	**10 ml/m^3 (ppm)** **40 mg/m^3**
MAK value dates from:	**1992**
Synonyms:	2-furancarbinol furfural alcohol furfuralcohol 2-furfuryl alcohol furyl alcohol α-furylcarbinol 2-furylcarbinol 2-furylmethanol 2-hydroxymethylfuran
Chemical name (CAS):	2-furanmethanol
CAS number:	98-00-0
Structural formula:	
Molecular formula:	$C_5H_60_2$
Molecular weight:	98.1
Melting point:	–31°C
Boiling point:	170 °C
Vapour pressure at 30 °C:	1.33 hPa
1 ml/m^3 (ppm) = 4.07 mg/m^3	**1 mg/m^3 = 0.25 ml/m^3 (ppm)**

1 Toxic Effects and Modes of Action

The toxic effects of furfuryl alcohol have not been studied in detail. The acute toxicity differs markedly in different species, the rat being most sensitive and the guinea pig least sensitive to the effects of the substance. The mechanism of the acute toxicity is not known. The effects seen in lethally intoxicated animals are unspecific and include spasms, respiratory failure and pulmonary haemorrhage and oedema. There are practically no studies of pharmacokinetics or metabolism of the substance, nor of chronic effects, reproductive toxicity, teratogenicity or carcinogenicity. Furfuryl alcohol

is not mutagenic. There are, however, indications of clastogenic activity. Furfuryl alcohol has irritant effects on the eyes; skin irritation has not been clearly documented.

1.1 Pharmacokinetics

After oral administration of furfuryl alcohol to rats, the main metabolite detected with UV spectroscopy was furoyl glycine (NIOSH 1979). After inhalation of the substance, the excretion of furancarboxylic acid was shown to be correlated with the exposure concentration (Savolainen and Pfäffli 1983).

There are no other studies of the pharmacokinetics of furfuryl alcohol.

2 Effects in Man

The odour threshold for furfuryl alcohol was determined in 13 test persons as 7–8 ml/m^3 (Jacobson *et al.* 1958).

Of 17 foundry workers subjected to lung function tests, 9 were shown to have reduced FEV_1 values. The furfuryl alcohol concentrations measured ranged up to 50 ml/m^3, formaldehyde concentrations were about 4 ml/m^3. Because of the presence of other substances and the relatively high formaldehyde concentrations, the effects on lung function could not be ascribed to the furfuryl alcohol exposures (Low and Mitchell 1985). In three test persons, oral administration of 0.6 to 1.0 g furfuryl alcohol as a 5 % aqueous solution resulted in an increase in respiration rate (Erdmann 1902).

Dermatitis and irritation of the respiratory passages was seen in bricklayers who came into contact with an acid-resistant cement containing furfuryl alcohol. The effects, however, could not be ascribed unambiguously to furfuryl alcohol (Mastromatteo 1965).

In a foundry in which a mixture of furfuryl alcohol, paraformaldehyde, phosphoric acid and sulfuric acid was poured onto hot sand, eye irritation with lacrimation developed after about 15 minutes in two persons exposed to a concentration of 15.8 ml/m^3 furfuryl alcohol; the formaldehyde concentration was 0.33 ml/m^3. At furfuryl alcohol concentrations of 8.6 and 10.8 ml/m^3, the irritation did not develop. It is, however, not clear which of the substances caused the eye irritation seen at the highest furfuryl alcohol concentration (NIOSH 1979). None the less, the American threshold limit value of 10 ml/m^3 is based on these findings (Federal Register 1989).

In an unpublished study, no evidence of eye irritation was seen in workers exposed during an 8-hour shift at a similar workplace to furfuryl alcohol concentrations up to 15 ml/m^3 (NIOSH 1979).

3 Effects on Animals

3.1 Acute toxicity

The oral LD_{50} for undiluted furfuryl alcohol in the rat is given as 110 to 132 mg/kg body weight. When the furfuryl alcohol was administered as a 2 % aqueous solution, the value was in the range between 275 and 451 mg/kg body weight. For the mouse, an oral LD_{50}

of about 2000 mg/kg body weight was determined (NIOSH 1979). For the rabbit, with a 10 % aqueous solution of furfuryl alcohol, the intravenous LD_{50} was 650 mg/kg body weight, with a 4% aqueous solution the subcutaneous LD_{50} was 796 mg/kg body weight. The subcutaneous LD_{50} for the rat was 78 mg/kg (undiluted) or 96 mg/kg (4% aqueous solution).

The 4-hour LC_{50} for the rat is given as 232 ml/m^3 furfuryl alcohol (Jacobson *et al.* 1958). After 6-hour exposures, the LC_{50} for the rat was 85 ml/m^3, and for the mouse 397 ml/m^3. In other studies with rats which inhaled 700 ml/m^3 furfuryl alcohol, the mortality was only 22% after a 4-hour exposure and 25 % after an 8-hour exposure. No deaths occurred in rabbits exposed to furfuryl alcohol concentrations up to 416 ml/m^3, in dogs up to 349 ml/m^3 or in monkeys up to 260 ml/m^3 (NIOSH 1979).

The symptoms of acute furfuryl alcohol intoxication are initial excitement, then sleepiness, respiratory depression, reduction in blood pressure, spasms and central respiratory depression. Autopsy revealed pulmonary haemorrhage and oedema, haematuria and congestion in the liver. After inhalation, eye irritation developed as well (NIOSH 1979).

Furfuryl alcohol had a negative inotropic effect on the isolated rabbit heart; this is indicative of direct effects on the myocardium (Fine and Wills 1950).

Effects on skin and mucous membranes

The LD_{50} after topical application of furfuryl alcohol to the shaved skin of rabbits was 657 mg/kg body weight. With the same application route, the LD_{50} for the mouse was 4920 mg/kg and for the guinea pig 8500 mg/kg. After four applications of furfuryl alcohol doses of 300 to 1400 mg/kg to the skin of rats, 11/12 animals died (NIOSH 1979). The symptoms of intoxication were like those after inhalation or oral administration, which indicates that the substance penetrates the skin. Application of 5.6 g (5 ml) furfuryl alcohol once daily to rabbit skin had no toxic effects (NIOSH 1979).

Application of 56 mg (0.05 ml) furfuryl alcohol to the rabbit eye resulted in inflammation, secretion of mucous and clouding of the cornea. The effects were reversible within 40 to 64 days. When 23 mg furfuryl alcohol was applied, the symptoms were less marked and recovery took place within 2 to 8 days (NIOSH 1979).

3.2 Subacute toxicity

After exposure of rats and mice to a furfuryl alcohol concentration of 19 ml/m^3, 6 hours daily, 5 times weekly for 6 or 3 weeks, no deaths occurred. The symptoms of toxicity were restlessness at the beginning of the exposures and then sleepiness during the whole of the exposure period. Other effects such as organ changes were not observed. No effects were seen in dogs exposed in the same way for 4 weeks to a furfuryl alcohol concentration of 239 ml/m^3 (NIOSH 1979).

3.3 Subchronic and chronic toxicity

In a study of the neurotoxicity of furfuryl alcohol, exposure of rats for 6 hours daily, 5 days per week to concentrations of 50 or 100 ml/m^3 resulted after 16 weeks in reduced

body weights and muscle hypotonus. In the brain there were signs of mitochondrial damage in the glia cells and first signs of demyelinization. After exposures to 25 ml/m^3, these effects did not develop (Savolainen and Pfäffli 1983). There are no studies of the chronic toxicity of furfuryl alcohol.

4 Genotoxicity

Furfuryl alcohol was not mutagenic in the Ames test in the *Salmonella typhimurium* strains TA98, TA100, TA1535 or TA1537 either with or without metabolic activation (Florin *et al.* 1980, Mortelmans *et al.* 1986). Nor was there any evidence for sister chromatid exchange in human lymphocytes incubated with furfuryl alcohol *in vitro* (Gomez-Arroyo and Souza 1985, Jansson *et al.* 1986). However, in CHO cells, concentration-dependent clastogenic activity of furfuryl alcohol was observed; it was increased by the addition of S9 mix (Stich *et al.* 1981).

Furfuryl alcohol was not mutagenic in *Drosophila melanogaster* (Rodriguez-Arnaiz *et al.* 1989).

5 Manifesto (MAK value, classification)

The data which were used for the establishment of the American threshold limit value cannot be used for the establishment of a MAK value because the eye irritation (lacrimation) which was observed in the exposed persons cannot be ascribed unambiguously to the furfuryl alcohol exposure. The situation is similar for skin irritation assumed to have been caused by furfuryl alcohol. Therefore the MAK value must be derived from the available animal studies. Since the LC_{50} for the most sensitive of the species which have been studied, the rat, is 85 ml/m^3, the no effect level for acutely toxic effects of furfuryl alcohol must be clearly below this level. Subacute exposure of rats under conditions comparable with workplace exposures produced only insignificant effects at a furfuryl alcohol concentration of 19 ml/m^3. Neurotoxic effects did not develop in rats after subchronic exposure to 25 ml/m^3 (NOEL). Since there are no studies of the relative sensitivities of man and rat to furfuryl alcohol, for safety the MAK value must be lower than this concentration. The MAK value for furfuryl alcohol is established at 10 ml/m^3. It requires further substantiation.

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completed 26.6.1992

ATTACHMENT D:

Rowland, J. et al. (2014) February 6, 2014, memorandum, "Furfural and Furfuryl Alcohol: Report of the Cancer Assessment Review Committee (CARC). OCSPP, US EPA 43 pp.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

DATE: February 6, 2014

SUBJECT: **FURFURAL and FURFURYL ALCHOHOL:** Report of the Cancer Assessment Review Committee

PC Code: 043301 and 643300	DP Barcode: N/A
Decision No.: N/A	Registration No.: N/A
Petition No.: N/A	Regulatory Action: N/A
Risk Assessment Type: Cancer Assessment	Case No.: N/A
TXR No.: 0056891	CAS No.: N/A
MRID No.: N/A	40 CFR: N/A

FROM: Jess Rowland, Co-Chair
Karlyn Middleton, Co-Chair
Cancer Assessment Review Committee
Health Effects Division (7509P)

TO: Elissa Reaves, Branch Chief (RABIV)
Health Effects Division (7509P)

The Cancer Assessment Review Committee (CARC) met on December 11, 2013 to evaluate the cancer classification of furfural and furfuryl alcohol in accordance with the *EPA's Final Guidelines for Carcinogen Risk Assessment* (March, 2005). Attached please find the final Cancer Assessment Document.

CANCER ASSESSMENT DOCUMENT

EVALUATION OF THE CARCINOGENIC POTENTIAL OF

Furfural and Furfuryl Alcohol

PC CODES: 043301 and 643300

FINAL
FEBRUARY 6, 2014

CANCER **A**SSESSMENT **R**EVIEW **C**OMMITTEE
HEALTH EFFECTS DIVISION
OFFICE OF PESTICIDE PROGRAMS

TABLE OF CONTENTS

EXECUTIVE SUMMARY ……4

I. Introduction 7

II. Background Information 7

III. Evaluation of Carcinogenicity Studies.....8

1. Carcinogenicity Study in Rats - Furfural 8

2. Carcinogenicity Study in Mice - Furfural 11

3. Carcinogenicity Study in Rats - Furfuryl Alcohol 17

4. Carcinogenicity Study in Mice - Furfuryl Alcohol.....24

IV. Toxicology.....28

1. Metabolism 28

2. Mutagenicity 29

3. Structure Activity Relationship.....35

4. Subchronic and Chronic Toxicity 37

5. Mode of Action Studies 38

V. Committee's Assessment of the Weight-of-the Evidence 39

VI. Classification of Carcinogenic Potential 41

VII. Quantification of Carcinogenic Potential 42

VIII. CARC Recommendations.....42

IX. References.....42

EXECUTIVE SUMMARY

On December 11, 2013, the Cancer Assessment Review Committee (CARC) of the Health Effects Division of the Office of Pesticide Programs evaluated the carcinogenic potential of furfural and furfuryl alcohol. Furfural is a pesticide active ingredient that is used to control root-infesting plant parasitic nematodes and fungal plant diseases and is currently registered for non-food use on athletic fields and outdoor ornamentals. In accordance with the 40 CFR Part 158 Toxicology Data requirements, long-term studies (i.e., chronic/carcinogenicity study in rats and the carcinogenicity study in mice) are not required for non-food use pesticides. The Agency has received a petition for a new use pattern (injection into soil) for pre-plant soil which may result in exposure via drinking water and thus there is a need for a dietary risk assessment. Consequently, toxicology and carcinogenicity studies conducted by the National Toxicology Program (NTP) were used to assess the carcinogenic potential of furfural/furfuryl alcohol. Additionally, all available data on toxicity, metabolism, mutagenicity, Structure Activity Relationships (SAR), and the report of the European Food Safety Authority (EFSA) on furfural derivatives were considered in this evaluation.

Male and female F344 N rats received oral (gavage) administration of furfural (99% pure) in corn oil at 0, 30 or 60 mg/kg/day for 104 weeks (MRID 46011016). Cholangiocarcinomas of the liver was seen in male rats at the high dose. The increase in the tumors incidences did not reach statistical significance when compared with the concurrent controls; however, historical control data indicate that this is a rare tumor and the incidences in this study (4%) were 10-fold higher than the historical control incidence (0.4%) for this tumor type. In addition, the presence of this tumor was corroborated by the occurrence of bile duct lesions which were histologically similar to the cholangiocarcinomas and were accompanied by fibrosis. These lesions were considered to be an early stage in the development of cholangiocarcinomas and thus could progress to this lesion. Also centrilobular necrosis of the liver occurred at increased incidences in the treated groups. The concern for this tumor was elevated since cholangiocarcinomas of the liver were also seen in male rats exposed to furan which is structurally related to furfural. Based on these considerations, the CARC concluded that the cholangiocarcinomas of the liver in male rats are treatment-related. The CARC considered the doses tested in both sexes to be adequate, but not excessive, to assess the carcinogenic potential of furfural. This determination was based on the results of the 16-day and 13-week studies which used for dose selection and the presence of non-neoplastic and neoplastic lesions in male rats in the main study.

Male and female B6C3F1 mice received oral (gavage) administration of furfural (99%) at 0, 50, 100 or 175 mg/kg/day in corn oil for 104 weeks (MRID 46011016). There were statistically significant ($p \leq 0.01$) increases in the incidences of hepatocellular adenomas, carcinomas, and combined adenomas and/or carcinomas at the high dose in males. The increase in carcinomas was particularly note worthy, with the incidence in the high dose group being three-fold greater than the incidence in the vehicle control group. In female mice, incidences of hepatocellular adenomas increased with dose and were significantly ($p \leq 0.01$) greater in the high dose. The incidences of all of these tumors exceeded the reported historical control ranges. The tumors were also corroborated by the presence of non-neoplastic lesions characterized as multifocal pigmentation and chronic inflammation of the subserosa of the liver in both sexes of mice. The concern for this tumor type was elevated since liver tumors were also seen in male

rats exposed to 5-methylfurfural, which is structurally related to furfural. Therefore, the CARC considered the liver tumors in male and female mice to be treatment-related. The doses used in the mouse cancer study were considered to be adequate, but not excessive, in both sexes, to assess the carcinogenic potential of furfural. This was based on the results of the 16-day and 13-week studies used for dose selection, and the presence of non-neoplastic lesions in both sexes of mice in the main study.

Male and female F344/N rats were exposed to furfuryl alcohol (98% pure) by inhalation (vapor) at concentrations of 0, 2, 8 or 32 ppm for 104 weeks (MRID 49161601). Nasal cavity tumors were seen in both sexes of rats. Nasal epithelial squamous cell carcinomas were seen in 11% of male rats at the highest concentration compared to none in the chamber controls. There were also significant ($p \leq 0.01$) increases in combined nasal epithelial carcinomas and epithelial squamous cell carcinomas at the high dose (12%) compared to chamber controls (0%). Female rats only had a significant trend ($p < 0.05$) for nasal epithelial adenomas at the highest concentration. The occurrence of these tumors was corroborated by the presence of non-neoplastic nasal lesions observed in male and female rats. The incidences of hyperplasia of the lateral wall of the nasal cavity, atrophy and metaplasia of the olfactory epithelium, and hyperplasia of the respiratory epithelium were significantly elevated relative to controls at all three exposure concentrations in both sexes. The lesions increased in incidence and severity with increasing concentration. The CARC considered the nasal tumors in male rats to be treatment-related. The doses used in the rat study were considered to be adequate, but not excessive, in both sexes to assess the carcinogenic potential of furfuryl alcohol. This was based on the results of a subchronic inhalation study used for dose selection and the presence of non-neoplastic lesions in both sexes in the main study.

Male and female B6C3F1 mice were exposed to furfuryl alcohol (98% pure) by inhalation (vapor) at concentrations of 0, 2, 8 or 32 ppm for 104 weeks (MRID 49161601). Kidney tumors were seen in male mice. There were statistically significant increases for renal adenomas (6%; trend at $p \leq 0.01$), carcinomas (4%) and combined adenomas/carcinomas (10% at $p \leq 0.05$) at the high dose when compared to controls (0%). Kidney tumors are rare among the historical controls and the incidences of the combined tumors in this study (10%) were 25-fold higher than the historical control incidence (0.4%). The tumors were corroborated by the presence of non-neoplastic kidney lesions in both male and female mice. The severity of the lesions increased with increasing dose in male mice only. Therefore, the CARC considered the kidney tumors in male mice to be treatment-related. The doses used in the mouse cancer study with furfuryl alcohol were considered to be adequate, but not excessive, in both sexes to assess the carcinogenic potential of furfuryl alcohol. This was based on the results of a subchronic inhalation study used for dose selection, and the presence of non-neoplastic lesions in both sexes and neoplastic lesions in males in the main study.

Based on the available NTP genetic toxicology data, there is no mutagenic concern for furfural or furfuryl alcohol. Overall, the data for furfural suggest that it has intrinsic mutagenic potential in cultured mammalian cells. However, it is not expressed in whole animals since it is rapidly metabolized by the liver and rendered either non-mutagenic or markedly less mutagenic. Additionally, the negative data for the *in vivo* gene mutations assay, which examined the mouse liver as the target for furfural-induced tumorigenic activity, rule out mutagenicity as a possible

mode of action for the induction of liver tumors seen in the 2-year mouse bioassay. Furfuryl alcohol is not mutagenic in bacteria and does not cause chromosome aberrations or sister chromatid exchange induction in mammalian cells. The *in vitro* data are supported by the results of whole animals studies showing that furfuryl alcohol was not clastogenic, aneugenic or genotoxic in mouse bone marrow cytogenetic, micronucleus or SCE assays.

An analysis of structure activity relationships for furfural and furfuryl alcohol, provide additional support for the carcinogenic potential of furfural. A rare tumor type (cholangiocarcinomas of the liver) seen in rats exposed to furfural was also seen in rats exposed to furan. Similarly, liver tumors seen in male mice with furfural were also seen in mice exposed to 5-methylfurfural. Being a direct-acting reactive electrophilic chemical, furfural is expected to have a greater potential for inducing cancer by the inhalation route than the oral route because of the portal-of-entry effect. A structurally related chemical, formaldehyde, is a well-known carcinogen via the inhalation route but does not seem to be have a cancer concern by the oral route unless there is a massive exposure to overwhelm the detoxification capacity. There is some suggestive evidence that the nasal carcinogenic effect of furfuryl alcohol via the inhalation route may be related to its oxidation to furfural as the proximate or ultimate carcinogen. Because of these concerns, the CARC recommends a chronic toxicity/carcinogenicity study in rats by the inhalation route be conducted for furfural.

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005): **Furfural is classified as "Likely to Be Carcinogenic to Humans"** based on the following considerations:

(i) Treatment-related cholangiocarcinoma of the liver, a rare tumor type, observed in male rats;
(ii) Treatment-related liver tumors (adenomas, carcinomas and/or combined adenomas/carcinomas) observed in male and female mice; and
(iii) Occurrence of hepatocellular neoplasms in each sex of mice with compounds structurally very similar to furfural

Furfuryl alcohol as "Likely to Be Carcinogenic to Humans" based on the following consideration:

(i) Treatment-related nasal tumors (adenomas, carcinomas and/or squamous cell carcinomas observed in male rats): and
(ii) Treatment-related kidney tumors (adenomas, carcinomas and/or combined adenomas/carcinomas observed in male mice.

The CARC recommended the low dose extrapolation method (Q_1*) for quantification of human cancer risk since no mode of action studies are available for the tumor types seen in animals treated with furfural or furfuryl alcohol.

I. INTRODUCTION

On December 11, 2013, the Cancer Assessment Review Committee (CARC) of the Health Effects Division of the Office of Pesticide Programs met to evaluate the carcinogenic potential of furfural and furfuryl alcohol.

II. BACKGROUND INFORMATION

Furfural is an aromatic aldehyde that is currently registered for non-food use on athletic fields and outdoor ornamentals. The current action is requesting a new use pattern (injection into soil) for pre-plant soil. Furfural (Figure 1) is a pesticide active ingredient that is used to control root-infesting plant parasitic nematodes and fungal plant diseases. The pesticidal mode of action is an interaction with the cuticle of the nematode, resulting in a stripping of the protective layers which results in the cuticle swelling and disintegration. This leads to decreased movement of the nematode and it subsequently dies through dehydration or attack by parasitic organisms. The mammalian mode of toxic action has not been identified. In its natural state, furfural is a liquid with high water solubility (s ≥ 74,100 ppm, FAO solubility classification) and is miscible with a variety of common organic solvents. Furfural has a relatively high vapor pressure and a moderately high Henry's Law constant (2.5 mm Hg and 4.4 x 10^{-6} atm-m^3/mol, respectively). Its C_{water}/C_{air} is equal to 5,812 (unitless), and its K_{AW} is 1.72×10^{-4}. These values indicate that furfural is slightly volatile from a water surface. The major furfural degradates, 2-furoic acid and furfuryl alcohol (Figure 2) may also be somewhat volatile (vapor pressures of 0.103 and 0.609 mmHg, respectively). Furfuryl alcohol is miscible with water. The octanol/water partition coefficients for furfural, 2-furoic acid and furfuryl alcohol 1 are very low (K_{OW} = 2.2, 4.4 and 1.9, respectively), indicating a very low tendency to bioaccumulate/bioconcentrate.

Figure 1. Structure of furfural.

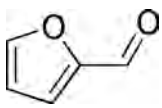
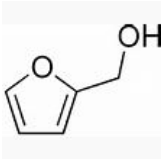


Figure 2. Structure of furfuryl alcohol.



III. EVALUATION OF CARCINOGENICITY STUDIES

At the present time, furfural is registered for non-food use on athletic fields and outdoor ornamentals. Since long-term toxicity and carcinogenicity studies are not required for non-food use chemicals, none were submitted to the Agency. However, the request for a new use may result in exposure via drinking water and thus a dietary assessment is required. Consequently, the toxicology and carcinogenicity studies with furfural conducted by the National Toxicology Program (NTP) will be used to evaluate the carcinogenic potential of furfural following oral (gavage) exposure (MRID 46011016) and furfuryl alcohol following inhalation exposures (MRID 49161601).

1. Carcinogenicity Study in Rats with Furfural

Reference: NTP (National Toxicology Program). 1990. NTP Technical Report on the Toxicology and Carcinogenesis Studies of Furfural (CAS No. 98-01-1) in F344/N rats and B6C3F1 mice (Gavage Studies). NTP-TR-382, MRID 46011016.

A carcinogenicity oral gavage study in F344/N rats was conducted by the NTP. In this study, F344/N male and female rats (50/sex/dose) were treated via gavage with 0, 30, or 60 mg/kg bw/day, in corn oil, 5 days/wk for 2 years (104 weeks). Rats were 7-8 weeks of age at the start of the study.

Survival data and tumors were evaluated by the Office of Pesticide Programs Health Effects Division the results of which are presented below. For further details, see TXR 0056717, Memorandum from L. Brunsman to A. Howard (July 25, 2013).

A. Experimental Design- Rat Study

Furfural was administered in corn oil to F344/N rats (50/sex/dose) at dose levels of 0, 30, or 60 mg/kg bw/day, 5 days/wk via oral gavage for 2 years (104 weeks) in a combined chronic toxicity/carcinogenicity study.

B. Discussion of Survival Data- Rat Study

Although 21 male rats died of accidental death (6 in the control group, 7 at 30 mg/kg/day and 8 at 60 mg/kg/day), there were no statistically significant survival disparities among the dose groups for male rats (Table 1). No statistical evaluation of survival was performed on the female rats, but 38% (19/50) of the female rats died of accidental death in the 60 mg/kg/day dose group. In comparison, there were only 4 accidental deaths in the female rats in the control group, and only 2 accidental deaths at 30 mg/kg/day. However, the mortality observed at the high dose was not considered excessive and the doses used in female animals were adequate for assessing carcinogenicity.

Table 1. Furfural – F344/N Rat Study Male Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (mg/kg/day)	Weeks				
	1-26	27-52	53-78	79-105[f]	Total
0	0/50	3/50	9/47	7/38	19/50 (38)
30	2/50	2/48	3/46	15/43	22/50 (44)
60	2/50	2/48	6/46	16/40	26/50 (52)

[+]Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
[f] Final sacrifice at weeks 104-105.
( ) Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

C. Neoplastic Lesions- Rat Study

There were no statistically significant tumor findings in male or female rats (no p values < 0.05). The statistical analyses of the liver cholangiocarcinomas in the male rats were based upon Exact test for trend and the Fisher's Exact Test (Table 2).

Table 2. Furfural – F344/N Rat Study Male Liver Tumor Rates[+] and Fisher's Exact Test and Exact Trend Test Results

Tumor Type	Dose (mg/kg/day)			Historical Control (HC)
	0	30	60	
Cholangiocarcinomas (%)	0/47 (0)	0/46 (0)	2[a]/46 (4)	SRI: 2/449 (0.4%) SD 0.88% Overall: 3/2,145 (0.1%) SD= 0.52%
p =	0.1079	1.0000	0.2419	

[+]Number of tumor bearing animals/Number of animals examined, excluding those that died before week 52.
[a]First cholangiocarcinoma observed at week 81, dose 60 mg/kg/day.
Historical Incidence of Bile Duct Neoplasms in Male F344/N Rats at Southern Research Institute (SRI): 2/449 (0.4%); SD = 0.88%, Overall Historical Incidence: 3/2,145 (0.1%); SD= 0.52% (NTP, 1990)
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

D. Non-Neoplastic Lesions- Rat Study

Mild liver toxicity in the form of centrilobular necrosis and bile duct dysplasia with fibrosis were observed in male rats administered furfural (Table 3). Bile duct dysplasia with fibrosis is considered to be an early stage in the development of cholangiocarcinomas. Hematology was not performed and no other clinical signs were reported.

Table 3. Furfural – F344/N Rat Study Male Non-Neoplastic Lesions in the Liver (2-year)

	Dose (mg/kg/day)		
	0	**30**	**60**
Survivors to Terminal Sacrifice	31	28	24
Liver: Centrilobular necrosis	2	8	12
Liver: Bile Duct Dysplasia with Fibrosis	0	0	2

E. Adequacy of Dosing for Assessment of Carcinogenicity

The dose levels for this study were selected based on the results of a 16-day and 13-week studies. As shown in Table 4, mortality occurred at 90 mg/kg/day and 180 mg/kg/day. Because of treatment-related mortality, 60 mg/kg/day was selected as the high dose for the bioassay. The CARC determined that the doses tested were adequate, but not excessive, in both sexes to assess the carcinogenic potential of furfural. This was based on the presence of non-neoplastic and neoplastic lesions in male rats. No changes in body weight were observed throughout the 2-year study. The NTP tested only two doses as opposed to the guideline requirement (Part 158 Test Guideline study 870.4200) of three treatment levels and a control group. The CARC determined that this study is adequate since: the doses were selected based on the findings of the 16-day and 13-week studies. Non-neoplastic lesions and evidence for carcinogenicity were seen at the high dose.

Table 4. Mortality data for male and female F344/N rats in the 13-week oral gavage study of furfural.

	Dose (mg/kg/day)					
	0	**11**	**22**	**45**	**90**	**180**
Males	1/10[a]	0/10	0/10	0/10	1/10[a]	9/10[b]
Females	1/10[a]	0/10	0/10	0/10	4/10[b]	10/10[c]

[a]Death gavage related
[b]3 deaths were gavage related
[c] 1 death was gavage related

F. NTP Conclusions

The NTP concluded that there was some evidence of carcinogenic activity in male F344/N rats and no evidence of carcinogenic activity for female F344/N rats.

2. Carcinogenicity Study in Mice with Furfural

A. Experimental Design- Mouse Study

Furfural was administered in corn oil to B6C3F1 mice (50/sex/dose) at dose levels of 0, 50, 100, or 175 mg/kg bw/day, 5 days/wk via oral gavage for 2 years (104 weeks).

B. Discussion of Survival Data- Mouse Study

There were no statistically significant trends for survival for male or female mice (Tables 5 and 6), but there was a survival disparity with a statistically significant pair-wise comparison of the 100 mg/kg/day mid-dose group with the controls for male mice. However, no statistically significant pair-wise comparison for survival for male mice was seen at the high dose (175 mg/kg/day).

Table 5. Furfural – B6C3F$_1$ Mouse Study Male Mortality Rates$^+$ and Cox or Generalized K/W Test Results

Dose (mg/kg/day)	Weeks				
	1-26	27-52	53-78	79-105^f	Total
0	2/50	0/48	5/48	8/43	15/50 (30)
50	2/50	2/48	4/46	14/42	22/50 (44)
100	1/50	0/49	5/49	20/44	26/50 (52)*
175	5/50	1/45	4/44	13/40	23/50 (46)

$^+$Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
^{f}Final sacrifice at weeks 104-105.
( ) Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 6. Furfural – B6C3F1 Mouse Study Female Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (mg/kg/day)	Weeks				
	1-26	27-52	53-78	79-105[f]	Total
0	1/50	0/49	6/49	10/43	17/50 (34)
50	0/50	2/50	9/48	11/39	22/50 (44)
100	0/50	1/50	9/49	11/40	21/50 (42)
175	0/50	1/50	5/49	12/44	18/50 (36)

[+]Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
[f]Final sacrifice at weeks 104-105.
( ) Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If [*], then $p < 0.05$. If [**], then $p < 0.01$.

C. Neoplastic Lesions- Mouse Study

Male mice had statistically significant trends, and statistically significant pair-wise comparisons of the high dose with the controls, for liver adenomas, carcinomas, and adenomas and carcinomas combined, all at $p < 0.01$. The increase in carcinomas was particularly noteworthy, with the incidence in the high dose group being three-fold greater than the incidence in the vehicle control group. The kidney tumor findings in the male rat were not statistically significant. The statistical analyses of the tumors in the male mice were based upon the Exact test for trend and the Fisher's Exact Test (Tables 7 and 8).

Female mice had significant trends for liver adenomas, liver adenomas and carcinomas combined, and forestomach squamous cell papillomas, all at $p < 0.01$. There was also a significant pair-wise comparison of the 175 mg/kg/day dose group with the controls for liver adenomas at $p < 0.05$. The statistical analyses of the tumors in the male mice were based upon the Exact test for trend and the Fisher's Exact Test (Tables 9 and 10).

Table 7. Furfural – B6C3F$_1$ Mouse Study Male Hepatocellular Tumor Rates[+] and Fisher's Exact Test and Exact Trend Test Results

Tumor Type	Dose (mg/kg/day)				
	0	50	100	175	Historical Control
Adenomas (%)	9/48 (19)	13[a]/46 (28)	11/48 (23)	19/44 (43)	SRI: 73/448 (16.3%) SD 9.51%
p =	0.00959**	0.19917	0.40105	0.00997**	Overall: 338/2,183 (15.5%) SD 7.01%
Carcinomas (%)	7/48 (15)	12[b]/46 (26)	6/48 (13)	21/44 (48)	SRI: 108/448 (24.1%), SD 7.16%
p =	0.00076**	0.12887	0.72385	0.00055**	Overall: 418/2,183 (19.1%), SD 7.42%
Combined (%)	16/48 (33)	22[c]/46 (48)	17/48 (35)	32[d]/44 (73)	SRI: 168/448 (34.5%) SD 9.57% Overall: 713/2,183 (32.7%), SD 8.55%
p =	0.00038**	0.11096	0.50000	0.00015**	

[+]Number of tumor bearing animals/Number of animals examined, excluding those that died before week 52.
[a]First adenoma observed at week 70, dose 50 mg/kg/day.
[b]First carcinoma observed at week 62, dose 50 mg/kg/day.
[c]Three animals in the 50 mg/kg/day dose group had both an adenoma and a carcinoma.
[d]Eight animals in the 175 mg/kg/day dose group had both an adenoma and a carcinoma.
Historical incidence of hepatocellular neoplasms in Male B6C3F1 mice at Southern Research Institute (SRI) Adenoma Total: 73/448 (16.3%), SD 9.51% ; Overall: 338/2,183 (15.5%), SD 7.01%; Carcinoma Total: 108/448 (24.1%) , SD 7.16%; Overall:418/2,183 (19.1%), SD 7.42%; Adenoma or Carcinoma Total: 168/448 (37.5%), SD 9.57%; Overall 713/2,183 (32.7%), SD 8.55%. (NTP, 1990)

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 8. Furfural – B6C3F$_1$ Mouse Study Male Renal Cortical Tumor Rates[+] and Fisher's Exact Test and Exact Trend Test Results

Tumor Type	Dose (mg/kg/day)				Historical Controls
	0	50	100	175	
Adenomas (%) p =	0/48 (0) 0.1777	0/46 (0) 1.0000	1[a]/48 (2) 0.5000	1[a]/44 (2) 0.4783	—
Carcinomas (%) p =	0/48 (0) 0.5054	1[b]/46 (2) 0.4894	0/48 (0) 1.0000	0/44 (0) 1.0000	—
Combined (%) p =	0/48 (0) 0.2790	1/46 (2) 0.4894	1/48 (2) 0.5000	1/44 (2) 0.4783	SRI: 0/448 Overall: 8/2,183

[+]Number of tumor bearing animals/Number of animals examined, excluding those that died before week 52.
[a]First adenoma observed at week 104 simultaneously in final sacrifice animals at doses 100 and 175 mg/kg/day.
[b]First carcinoma observed at week 105 in a final sacrifice animal, dose 50 mg/kg/day.
Historical Incidence of renal cortical neoplasm in male B6C3F1 mice at Southern Research Institute (SRI):0/448 SD –not reported; Overall: 8/2,183 SD – not reported. (NTP, 1990)
Note: There were no statistically significant tumor findings in the kidney.

Table 9. Furfural – B6C3F1 Mouse Study Female Hepatocellular Tumor Rates[+] and Fisher's Exact Test and Exact Trend Test Results

Tumor Type	Dose (mg/kg/day)				Historical Control
	0	50	100	175	
Adenomas (%) p =	1/49 (2) 0.0056**	3/48 (6) 0.3008	5[a]/49 (10) 0.1021	8/49 (16) 0.0154*	SRI 17/450 (3.8%) SD 2.73% Overall 104/2,188 (4.8%) SD 3.96%
Carcinomas (%) p =	4/49 (8) 0.3528	0/48 (0) 1.0000	2/49 (4) 0.8980	4[b]/49 (8) 0.6426	SRI 11/450 (2.4%) SD 2.40% Overall 60/2,188 (2.7%) SD 2.41%
Combined (%) p =	5/49 (10) 0.0095**	3/48 (6) 0.8592	7/49 (14) 0.3796	12/49 (24) 0.0538	SRI 28/450 (6.2%) SD 2.33% Overall 162/2,188 (7.4%) SD 4.98%

[+]Number of tumor bearing animals/Number of animals examined, excluding those that died before week 52.
[a]First adenoma observed at week 70; [b]First carcinoma observed atweek 78.
Historical incidence of hepatocellular neoplasms in female B6C3F1 mice at Southern Research Institute (SRI). Adenoma Total: 17/450 (3.8%), SD 2.73%; Overall: 104/2,188 (4.8%) , SD 3.96%; Carcinoma Total: 11/450 (2.4%), SD 2.40%; Overall: 60/2,188 (2.7%), SD 2.41 %; Adenoma or Carcinoma Total: 28/450 (6.2%), SD 2.33%; Overall: 162/2,188 (7.4%), SD 4.98%. (NTP, 1990)
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 10. Furfural – B6C3F1 Mouse Study- Female Forestomach Tumor Rates[+] and Fisher's Exact Test and Exact Trend Test Results

Tumor Type	Dose (mg/kg/day)				Historical Control
	0	50	100	175	
Papillomas (%)	1/49 (2)	0/48 (0)	1[a]/49 (2)	6/49 (12)	SRI: 8/446 (1.8%) SD 2.73% Overall: 34/2,144 (1.6%) SD 2.74%
p =	0.00328**	1.00000	0.75258	0.05574	

[+]Number of tumor bearing animals/Number of animals examined, excluding those that died before week 52.
[a]First adenoma observed at week 75, dose 100 mg/kg/day.
Historical incidence of forestomach squamous cell neoplasm in female B6C3F1 mice at Southern Research Institute (SRI) – Papilloma Total: 8/446 (1.8%), SD 2.73%; Overall: 34/2,144 (1.6%), SD 2.74%; Papilloma or Carcinoma Total: 8/446 (1.8%), SD 2.73%,; Overall: 37/2,144 (1.7%), SD 2.74%.(NTP, 1990)
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

D. Non-Neoplastic Lesions- Mouse Study

Liver toxicity, in the form of chronic inflammation and increased hepatic pigmentation in male and female mice, was observed in the 2-year study (Table 11).

Table 11. Incidence data of non-neoplastic lesions in B6C3F1 mice in the 2-year oral gavage study of furfural.

	Dose (mg/kg/day)			
	0	**50**	**100**	**175**
Lesions Type	**Males**			
Liver Pigmentation	0/50	0/50	8/49	18/50
Chronic Inflammation	0/50	0/50	8/49	18/50
	Females			
Liver Pigmentation	0/50	0/50	0/50	11/50
Chronic Inflammation	0/50	0/50	1/50	8/50

E. Adequacy of Dosing for Assessment of Carcinogenicity

The dose levels for this study were selected based on the results of a 13-week study. As shown in Table 12, mortality occurred at the 600 and 1200 mg/kg/day dose levels. As shown in Table 13, liver lesions were also seen at these doses, as well as at 300 mg/kg/day. Based on these findings, 175 mg/kg/day was selected as the highest dose for the carcinogenicity study. The CARC considered the doses tested adequate, but not excessive, in both sexes to assess the carcinogenic potential of furfural. This was based on the presence of non-neoplastic lesions in the liver.

Table 12. Mortality data for B6C3F1 mice in the 13-week oral gavage study of furfural

	Dose (mg/kg/day)					
	0	75	150	300	600	1200
Males	0/10	0/10	0/10	0/10	9/10	10/10
Females	1/10[d]	0/10	9110[d]	0/10	9/10	10/10

[d] Death was gavage related

Table 13. Incidence data of non-neoplastic lesions for B6C3F1 mice in the 13-week oral gavage study of furfural.

	Dose (mg/kg/day)					
	0	75	150	300	600	1200
	Males					
Centrilobular Coagulative Necrosis	0/10	0/10	1/10	1/10	9/10	8/10
	Females					
Centrilobular Coagulative Necrosis	0/10[d]	0/10	0/10[d]	0/10	/10	2/10

F. NTP Conclusions

NTP concluded that there was clear evidence of carcinogenic activity in male $B6C3F_1$ mice and some evidence of carcinogenic activity in female $B6C3F_1$ mice.

Furfuryl Alcohol

Reference: NTP (National Toxicology Program). 1999. NTP Technical Report on the Toxicology and Carcinogenesis Studies of Furfuryl Alcohol (CAS No. 98-00-0) in F344/N rats and B6C3F1 mice (Inhalation Studies). NTP-TR-482, MRID 49161601.

A carcinogenicity inhalation study in F344/N rats was conducted by the NTP. In this study, F344/N male and female rats (50/sex/dose) were treated via inhalation with 0, 2, 8, or 32 ppm for 6 hours/day, 5 days/wk for 2 years (105 weeks). Rats were approximately 6 weeks of age at the start of the study.

Survival data and tumors were evaluated by the Office of Pesticide Programs Health Effects Division the results of which are presented below. For further details, see TXR 0056739, Memorandum from L. Brunsman to A. Howard (September 3, 2013).

3. Carcinogenicity Study in Rats - Furfuryl Alcohol

A. Experimental Design- Rat Study

Furfuryl alcohol was administered via inhalation to F344/N rats (50/sex/concentration) at concentrations of 0, 2, 8, or 32 ppm, 5 days/wk for 2 years (104 weeks) in a carcinogenicity study.

B. Discussion of Survival and Tumor Data

There were statistically significant survival disparities among the dose groups for male rats, as no male rats in the high dose group survived to termination (Table 14). However, the majority of the deaths in males at 32 ppm occurred after study week 91. Survival of all other exposed groups of male rats was similar to that of the chamber control group. Although the cause of death for the males at the high dose was not reported, nephropathy, a common spontaneous renal disease of F344 rats, was increased in males at 32 ppm (severity level, 3.7) compared to chamber controls (2.9). Additionally, males exposed to 32 ppm also exhibited an increased incidence of lesions related to kidney failure including parathyroid gland hyperplasia (39/50 vs. 9/49 in the controls ; P<0.01) and fibrous osteodystrophy (34/50 vs. 2/50 <p.0.01). There were no significant survival disparities for female rats (Tables 15).

Table 14. Furfuryl Alcohol – F344/N Rat Study Male Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (ppm)	Weeks				
	1-26	27-52	53-78	79-105[f]	Total
0	1/50	3/49	8/46	30/38	42/50 (84) **
2	0/50	0/50	10/50	35/40	45/50 (90)
8	0/50	0/50	11/50	30/39	41/50 (82)
32	0/50	1/50	9/49	40/40	50/50 (100) **

[+]Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
[f]Final sacrifice at week 105.
( ) Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 15. Furfuryl Alcohol – F344/N Rat Study Female Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (ppm)	Weeks				
	1-26	27-52	53-78	79-105[f]	Total
0	1/50	0/49	4/49	19/45	24/50 (48)*
2	0/50	0/50	6/50	18/44	24/50 (48)
8	0/49	0/49	5/49	22/44	27/49 (55)
32	0/50	1/50	8/49	25/41	34/50 (68)*

+Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
[f]Final sacrifice at week 105.
( ) Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

C. Neoplastic Lesions- Rat Study

Male rats had a significant trend for nasal epithelial squamous cell carcinomas at $p < 0.05$. There was also a significant trend at $p < 0.01$, and a significant pair-wise comparison of the high dose group with the controls at $p < 0.05$, for combined nasal lateral wall adenomas, epithelial adenomas, epithelial carcinomas and epithelial squamous cell carcinomas in male rats. The statistical analyses of the nasal lateral wall and nasal epithelial tumors in the male rats were based upon Peto's prevalence test (Table 16).

Female rats had a significant trend for nasal epithelial adenomas at $p < 0.05$. There were no statistically significant pair-wise comparisons of the dosed groups with the controls. Ad hoc analyses were run on the female rat kidney tumors because there was no individual animal data provided for the step sectioning performed on this tissue. The statistical analyses of the nasal and kidney tumors in female rats were based upon the Peto's prevalence test (Tables 17 and 18).

Table 16. Furfuryl Alcohol – F344/N Rat Study Male Nasal Tumor Rates[+] and Peto's Prevalence Test Results

Tumor Type	Dose (ppm)				Historical Control (NTP, 1999)
	0	2	8	32	
Lateral wall Adenomas (%) p =	0/9 (0) 0.61596	1[a]/8 (13) 0.28185	0/10 (0) NA	0/0 (0) NA	SRI: 1/897 (0.1%) SD 0.5%
Epithelial Adenomas (%) p =	0/34 (0) 0.60688	0/34 (0) NA	1[b]/34 (3) 0.15391	0/33 (0) NA	
Epithelial Carcinoma (%) p =	0/30 (0) 0.06097	0/32 (0) NA	0/30 (0) NA	1[c]/28 (4) 0.18770	0/897
Epithelial Squamous Cell Carcinomas (%) p =	0/30 (0) 0.01085*	0/32 (0) NA	0/30 (0) NA	3[d]/28 (11) 0.09177	0/897
Combined (%) p =	0/34 (0) 0.00643**	1/34 (3) 0.28185	1/34 (3) 0.15866	4/33 (12) 0.03682*	—

[+]Number of tumor bearing animals/Number of animals examined, excluding those that died before observation of the first tumor.
[a]First Lateral wall Adenoma observed at week 103, dose 2 ppm
[b]First Epithelial Adenoma observed at week 84, dose 8 ppm
[c]First Epithelial Carcinoma observed at week 87, dose 32 ppm

[d]First Epithelial Squamous Cell Carcinoma observed at week 87, dose 32 ppm
Historical incidence of nasal neoplasms in chamber control male F344/N rats at Southern Research Institute (SRI) – Adenoma Total: 1/897 (0.1%), SD 0.5%; Carcinoma: 0/897; Squamous Cell Carcinoma: 0/897.(NTP, 1999)
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 17. Furfuryl Alcohol – F344/N Rat Study Female Nasal Tumor Rates+ and Peto's Prevalence Test Results

Tumor Type	Dose (ppm)				Historical Control (NTP, 1999)
	0	2	8	32	
Lateral Wall Adenomas	0/44	0/45	1[a]/46	0/41	Overall Total: 1/892 (0.1%) SD 0.5%
(%)	(0)	(0)	(2)	(0)	
p =	0.61881	NA	0.19324	NA	
Epithelial Adenomas	0/26	0/26	0/22	1[b]/16	
(%)	(0)	(0)	(0)	(6)	
p =	0.01897*	NA	NA	0.10120	
Combined	0/44	0/45	1/46	1/41	—
(%)	(0)	(0)	(2)	(2)	
p =	0.12813	NA	0.19324	0.10120	

+Number of tumor bearing animals/Number of animals examined, excluding those that died before observation of the first tumor.
[a]First Lateral Wall Adenoma observed at week 78, dose 8 ppm
[b]First Epithelium Adenoma observed at final sacrifice week 105, dose 32 ppm
Historical incidence of nasal adenoma in chamber control female F344/N rats at Battelle Pacific Northwest Labs — Overall Total- 1/892 (0.1%) SD 0.5% (NTP, 1999).
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 18. Furfuryl Alcohol – F344/N Rat Study Female Renal Tubule Tumor Rates[+] and *ad hoc* Fisher's Exact Test and Exact Trend Test Results

Tumor Type	Dose (ppm)				Historical Control (NTP, 1999)
	0	2	8	32	
Adenomas (%) p =	0/50 (0) 0.0736	0/49 (0) 1.0000	2/49 (4) 0.2424	2/50 (4) 0.2475	Overall 1/898 (0.1%) SD 0.5%
Carcinoma (%) p =	0/50 (0) 0.5000	1/49 (2) 0.4950	0/49 (0) 1.0000	0/50 (0) 1.0000	Overall 4/898 (0.5%) SD 0.9%
Combined (%) p =	0/50 (0) 0.1421	1/49 (2) 0.4950	2/49 (4) 0.2424	2/50 (4) 0.2475	Overall 5/898 (0.6%) SD 0.9%

[+]Number of tumor bearing animals/Number of animals examined.
Historical incidence of renal tubule neoplasms in chamber control female F344/N rats at Battelle Pacific Northwest Labs – Adenoma Overall Total -1/898 (0.1%) SD 0.5%; Carcinoma – 4/898 (0.5%) SD 0.9%; Adenoma and Carcinoma – 5/898 (0.6%) SD 0.9% (NTP, 1999).
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

D. Non-Neoplastic Lesions- Rat Study

The primary target organs of the test substance for non-neoplastic effects were the nose and the kidney. The non-neoplastic microscopic lesions observed in the nose of male and female rats are summarized in Table 19. Furfuryl alcohol exposed groups had increased incidences of non-neoplastic lesions in the nose relative to those in controls. The incidences of hyperplasia of the lateral wall, atrophy and metaplasia of the olfactory epithelium, and hyperplasia of the respiratory epithelium were statistically elevated relative to controls at all three exposure concentrations in both sexes (typically $p \leq 0.01$). The incidences of nasal lesions increased with increasing exposure concentration, the most marked increase occurring between 2 and 8 ppm in both sexes. All animals exposed at 32 ppm were affected. The severity also increased with increasing concentration to a similar extent in both males and females. Necrosis was not observed in either sex, demonstrating that the basic structure of the nasal turbinates and mucosal lining remained intact. However, somewhat greater toxicity was evident in males with higher incidence rates. For example, fibrosis of the olfactory epithelium in males occurred in 1/50, 26/50, and 40/50 at 2, 8, and 32 ppm, respectively, whereas the incidences in females were 0/50, 16/50, and 31/50. The incidences of hyperplasia and squamous metaplasia, adaptive responses to chronic irritation, when not similar in males and females, were higher in males. The squamous metaplasia of the respiratory epithelium in males was 3/50 and 26/50 at 8 and 32 ppm, while the respective incidences in females were 2/50 and 10/50.

TABLE 19: Incidences of selected non-neoplastic lesions of the nose in rats exposed for 2 years to furfuryl alcohol.

	Dose (ppm)			
	0	2	8	32
Males -Number Examined	**50**	**50**	**50**	**50**
Suppurative inflammation[a]	3 (1.0)[b]	6 (1.5)	17** (1.7)	44** (2.1)
Glands, hyperplasia	0	0	22** (1.0)	49** (2.3)
Lateral wall, hyperplasia	1 (1.0)	49** (1.5)	50** (2.4)	50** (3.5)
Lateral wall, squamous metaplasia	1 (1.0)	1 (1.0)	8* (1.1)	33** (1.3)
Olfactory epithelium, atrophy	1 (1.0)	12** (1.1)	47** (1.8)	50** (2.4)
Olfactory epithelium, hyaline degeneration	42 (1.3)	48 (1.5)	50** (2.6)	47 (2.7)
Olfactory epithelium, fibrosis	0	1 (1.0)	26** (1.0)	40** (2.0)
Olfactory epithelium, hyperplasia	0	1 (1.0)	42** (1.0)	40** (1.8)
Olfactory epithelium, metaplasia	1 (1.0)	8* (1.3)	37** (1.5)	49** (2.2)
Respiratory epithelium, hyaline degeneration	12 (1.0)	14 (1.0)	45** (1.6)	3* (1.7)
Respiratory epithelium, hyperplasia	0	26** (1.8)	50** (2.5)	50** (3.5)
Respiratory epithelium, squamous metaplasia	0	0	3 (1.0)	26** (1.4)
Females -Number Examined	**49**	**50**	**48**	**49**
Suppurative inflammation[a]	4 (2.3)	1 (2.0)	5 (1.4)	23** (1.7)
Glands, hyperplasia	0	0	24** (1.0)	46** (2.2)
Lateral wall, hyperplasia	0	39** (1.3)	48** (2.1)	49** (3.3)
Lateral wall, squamous metaplasia	0	1 (1.0)	0	24** (1.0)
Olfactory epithelium, atrophy	0	6* (1.3)	44** (1.7)	49** (2.3)
Olfactory epithelium, hyaline degeneration	43 (1.2)	50* (1.6)	47 (2.7)	48 (3.3)
Olfactory epithelium, fibrosis	0	0	16** (1.3)	31** (1.7)
Olfactory epithelium, hyperplasia	0	0	31** (1.2)	41** (1.5)
Olfactory epithelium, metaplasia	0	5* (1.2)	37** (1.5)	48** (2.2)
Respiratory epithelium, hyaline degeneration	23 (1.0)	39** (1.2)	45** (1.9)	6** (2.0)
Respiratory epithelium, hyperplasia	0	18** (1.4)	40** (2.1)	49** (3.2)
Respiratory epithelium, squamous metaplasia	0	0	2 (1.0)	10** (1.2)

*Significantly different ($p<0.05$) from chamber control group by Poly-3 test ** $p \leq 0.01$
[a]Number of animals with lesions
[b]Average severity grade of lesion in (); 1=minimal, 2=mild, 3=moderate, 4=marked

E. Adequacy of Dosing for Assessment of Carcinogenicity

The concentrations tested were selected based on the results of a 14-week inhalation study. Incidences and severities of cytological alterations in the nasal mucosa were associated with exposure to furfuryl alcohol at concentrations of 8 ppm or greater. Based on the histopathological lesions of the nasal mucosa, 32 ppm was selected as the high concentration for the bioassay. The CARC determined that the concentrations tested in the study were adequate in both sexes to assess the carcinogenic potential of furfuryl alcohol. This was based on the presence of non-neoplastic and neoplastic lesions of the nasal cavity. Mean body weights of 32 ppm males were less than those of the chamber controls beginning at week 19 (94% of control values); by week 91, the body weights of 32 ppm males were 76% of those of the chamber controls. Mean body weights of 2 and 8 ppm male groups and all exposed female groups were similar to those of the chamber controls, throughout the study. Although there was 100% mortality at the high dose, the CARC did not consider this concentration to be excessive, since survival was 80% at 18 months and 32 % at week 91 which met the test guideline requirement (i.e., survival no less than 50% at 18 months for rats). Additionally all rats were available for histopathological examination (i.e., no more than 10 percent of any group was lost due to autolysis, cannibalism, or management problems, as required by the test guideline).

F. NTP Conclusions

The NTP concluded that there was some evidence of carcinogenic activity of furfuryl alcohol in male rats and equivocal evidence of carcinogenic activity of furfuryl alcohol in female rats.

4. Carcinogenicity Study in Mice - Furfuryl Alcohol

A. Experimental Design- Mouse Study

Furfuryl alcohol was administered via inhalation to B6C3F1 mice (50/sex/dose) at concentrations of 0, 2, 8, or 32 ppm, 5 days/wk for 2 years (104 weeks) in a combined carcinogenicity study.

B. Discussion of Survival Data- Mouse Study

There were no statistically significant survival disparities among the dose groups in male or female mice (Table 20 and Table 21, respectively).

Table 20. Furfuryl Alcohol – B6C3F$_1$ Mouse Study Male Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (ppm)	Weeks				
	1-26	27-52	53-78	79-105	Total
0	0/50	0/50	6/50	10/44	16/50 (32)
2	0/50	2/50	2/48	10/46	14/50 (28)
8	0/50	2/50	9/48	9/39	20/50 (40)
32	0/50	0/50	2/50	10/48	12/50 (24)

[+]Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
[f]Final sacrifice at week 105.
( ) Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If [*], then $p < 0.05$. If [**], then $p < 0.01$.

Table 21. Furfuryl Alcohol – B6C3F$_1$ Mouse Study Female Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (ppm)	Weeks				
	1-26	27-52	53-78	79-105	Total
0	0/50	1/50	3/49	12/46	16/50 (32)
2	0/49[a]	1/49	5/48	9/42[b]	15/48 (31)
8	0/50	0/50	3/50	14/46[b]	17/49(35)
32	0/50	0/50	0/50	10/50	10/50 (20)

[+]Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
[f]Final sacrifice at week 105.
[a]One female in the 2 ppm dose group was found to be pregnant and removed from the study.

[b]Two accidental deaths, both at week 98, one each in the 2 and 8 ppm dose groups.
( ) Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

C. Neoplastic Lesions- Mouse Study

Male mice had significant trends for renal tubule adenomas at $p < 0.05$. There was also a significant trend at $p < 0.01$, and a significant pair-wise comparison of the 32 ppm dose group with the controls at $p < 0.05$, for renal tubule adenomas and/or carcinomas combined. The statistical analyses of the kidney tumors in male mice were based upon the ad hoc Exact test for trend and the Fisher's Exact Test (Table 22). Ad hoc analyses were run on the male mouse kidney tumors because there was no individual animal data provided for the step sectioning performed on this tissue. There was no evidence of carcinogenic activity with furfuryl alcohol in female mice.

Table 22. Furfuryl Alcohol – B6C3F$_1$ Mouse StudyMale Renal Tubule Tumor Rates[+] and *ad hoc* Fisher's Exact Test and Exact Trend Test Results

Tumor Type	Dose (ppm)				Historical Control (NTP, 1999)
	0	2	8	32	
Adenomas (%) p =	0/50 (0) 0.01538*	0/49 (0) 1.00000	0/49 (0) 1.00000	3/50 (6) 0.12121	Overall: 3/1,093 (0.3%) SD 0.6%
Carcinomas (%) p =	0/50 (0) 0.06281	0/49 (0) 1.00000	0/49 (0) 1.00000	2/50 (4) 0.24747	Overall: 1/1,093 (0.1%) SD 0.4%
Combined (%) p =	0/50 (0) 0.00088**	0/49 (0) 1.00000	0/49 (0) 1.00000	5/50 (10) 0.02814*	Overall: 4/1,093 (0.4%) SD 1.0%

[+]Number of tumor bearing animals/Number of animals examined.
Historical incidence of renal tubule neoplasms in chamber control male B6C3F1mice at Battelle Pacific Northwest Labs – Adenoma Overall Total -3/1,093 (0.3%) SD 0.6%; Carcinoma – 1/1,093 (0.1%) SD 0.4%; Adenoma and Carcinoma – 4/1,093 (0.4%) SD 1.0% (NTP, 1999).
Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

D. Non-Neoplastic Lesions- Mouse Study

The primary target organs of the test substance for non-neoplastic effects in mice were the nose, the kidney, and the eye (female mice only). In the nose, there were a variety of non-neoplastic histological changes which were statistically significantly increased in the treated groups, vs. chamber controls. The non-neoplastic lesions observed in the nose of male and female mice are summarized in Table 23. The severity of the lesions increased with increasing dose in both males and females and 3/50 females and 1/50 males at 32 ppm had necrosis of the respiratory epithelium. The change was not statistically significant, but does suggest that there is an increase in severity with increasing dose.

In the kidney, nephropathy was observed in all groups of male and female mice (summarized in Table 24). The severity increased with increasing dose in male mice only. The nephropathy consisted of necrosis and regeneration of the renal tubule epithelium and inflammation and fibrosis in the interstitium. The incidence of renal tubule degeneration in male mice exposed to 32 ppm was greater (statistically significantly) than in the controls. Additionally, renal tubule degeneration was observed and consisted of slightly distended tubules with lumens containing eosinophilic, finely granular material. Some degenerate tubules contained one to a few enlarged epithelial cells with large, sometimes pleomorphic nuclei.

There was a high incidence of degeneration of the cornea in female mice only, at 32 ppm (3/49, 1/49, 4/49, 26/50; at 0, 2, 8, and 32 ppm, respectively). Corneal degeneration consisted of mineralization of the stroma beneath the corneal epithelium.

Table 23. Incidences of selected non-neoplastic lesions of the nose in mice exposed for 2 years to furfuryl alcohol.

	Dose (ppm)			
	0	2	8	32
Males -Number Examined	**50**	**49**	**49**	**50**
Suppurative inflammation[a]	7 (1.4)[b]	11 (1.2)	27** (1.3)	28** (1.7)
Glands, hyperplasia	0	10** (1.0)	48** (1.8)	46** (3.3)
Glands, squamous metaplasia	0	6* (1.0)	35** (1.1)	47** (1.5)
Lateral wall, squamous metaplasia	0	9** (1.0)	10** (1.7)	20** (1.5)
Olfactory epithelium, atrophy	3 (1.0)	15** (1.2)	49** (2.2)	50** (3.6)
Olfactory epithelium, hyaline degeneration	2 (1.5)	3 (1.7)	21** (1.3)	39** (2.0)
Olfactory epithelium, metaplasia	0	12** (1.1)	49** (2.1)	50** (3.5)
Respiratory epithelium, hyaline degeneration	5 (1.0)	18** (1.1)	42** (1.3)	45** (1.2)
Respiratory epithelium, squamous metaplasia	0	2 (1.0)	10** (1.1)	20** (1.4)

	Dose (ppm)			
	0	2	8	32
Respiratory epithelium, necrosis	1 (2.0)	0	0	1 (1.0)
Respiratory epithelium, regeneration	0	1 (1.0)	13** (1.0)	21** (1.0)
Females -Number Examined	**50**	**48**	**49**	**50**
Suppurative inflammation	5 (1.2)	12* (1.1)	25** (1.5)	42** (2.0)
Glands, hyperplasia	0	33** (1.1)	46** (2.8)	47** (3.1)
Glands, squamous metaplasia	1 (1.0)	1 (1.0)	34** (1.1)	46** (1.5)
Lateral wall, squamous metaplasia	3 (1.0)	14** (1.4)	16** (1.4)	36** (1.9)
Olfactory epithelium, atrophy	2 (1.0)	35** (1.2)	49** (3.0)	50** (3.6)
Olfactory epithelium, hyaline degeneration	7 (1.3)	14 (1.4)	28** (1.8)	45** (2.2)
Olfactory epithelium, metaplasia	0	31** (1.2)	49** (3.0)	49** (3.6)
Respiratory epithelium, hyaline degeneration	19 (1.4)	44** (1.5)	49** (1.3)	48** (1.4)
Respiratory epithelium, squamous metaplasia	1 (1.0)	9** (1.8)	21** (1.7)	39** (1.9)
Respiratory epithelium, necrosis	0	0	2 (2.5)	3 (1.3)
Respiratory epithelium, regeneration	0	0	9** (1.0)	13** (1.2)

*Significantly different ($p<0.05$) from chamber control group by Poly-3 test

** $p\leq 0.01$

[a]Number of animals with lesions

[b]Average severity grade of lesion in (); 1=minimal, 2=mild, 3=moderate, 4=marked

Table 24. Incidences of Non-neoplastic Lesions of the Kidney in B6C3F1 mice exposed for 2 years to furfuryl alcohol. .

	Dose (ppm)			
	0	2	8	32
Males -Number Examined	**50**	**49**	**49**	**50**
Nephropathy	49 (1.2)	48 (1.4)	43 (1.5)	47 (1.8)
Renal Tubule, Degeneration	0	0	1 (1.0)	48**(1.0)
Renal Tubule, Hyperplasia	1 (1.0)	3 (1.0)	0	3 (1.7)
Females -Number Examined	**50**	**48**	**49**	**49**
Nephropathy	41 (1.0)	35 (1.1)	40 (1.2)	39 (1.0)

** $p\leq 0.01$; Average severity grade of lesion in (); 1=minimal, 2=mild, 3=moderate, 4=marked

E. Adequacy of Dosing for Assessment of Carcinogenicity

Exposure to concentrations of furfuryl alcohol (via inhalation) at up to 32 ppm for 14 weeks, produced few overt signs of toxicity in mice. No effects were observed on body weight in male or female mice. The most significant response occurred in the nose where a spectrum of inflammatory, degenerative, and proliferative lesions of the respiratory and olfactory epithelium were observed following exposure to furfuryl alcohol vapor. In mice, nasal lesions of the olfactory and respiratory epithelium were observed in all exposed groups. Changes were more severe in the anterior portion than in the posterior portion of the nose and severity increased with increasing exposure concentration. The integrity of the nasal mucosa or nasal passages was not disrupted, however. Therefore, for the cancer study, the concentrations of 2, 8, and 32 ppm were selected. The CARC determined that the highest concentration tested (32 ppm) was considered to be adequate, but not excessive, in both sexes to assess the carcinogenic potential of furfuryl alcohol. This was based on the presence of non-neoplastic and neoplastic lesions in the kidneys. Mean body weights of exposed males were generally similar to those of the chamber control group throughout the study. Mean body weights of females exposed to 2, 8, or 32 ppm were less than those of the chamber control group beginning at weeks 59, 59, or 39, respectively. Hematology was not performed.

F. NTP Conclusions

The NTP stated that there was some evidence of carcinogenic activity of furfuryl alcohol in male mice and no evidence of carcinogenic activity of furfuryl alcohol in female mice.

IV. TOXICOLOGY

1. Metabolism

A. Mammalian Metabolism of Furfural

No guideline metabolism studies are available for furfural. However, a study was identified in the open scientific literature that is considered acceptable for regulatory use. In this study, furfural was administered via gavage (in corn oil) and was rapidly absorbed and excreted, with about 80% of the elimination occurring within 24 hours. The major route of excretion was in the urine, in which 85% (out of a total recovered dose of 90%) was found by 72 hours. There were no changes in excretion indicative of saturation of excretion with increasing dose. Expired radioactivity (as carbon dioxide) was a minor route of excretion at 6.6% and was measured for the high dose only. The feces were also a minor route of excretion at ≤2% of the administered dose. Furfural was retained in tissues at low levels of less than 1% of the administered dose (range 0.1 ± 0.1% at 0.127 mg/kg to 0.6 ± 0.1% at 12.5 mg/kg), indicating low potential for bioaccumulation.

Furoylglycine was the major urinary metabolite for both the high and low dose groups, comprising over 75% of urinary metabolites by 48 hours. Furoic acid and furanacrylic acid were minor urinary metabolites that were present at <5% after 48 hours. The average levels of unidentified urinary metabolites were low, at less than 2%.

These results support a metabolic pathway in which furfural is converted to furanacrylic acid (presumably by condensation with acetyl-CoA), which is excreted in the urine (a minor pathway) or oxidized to furoic acid (the major pathway). Furoic acid can be excreted unchanged in the urine (a minor pathway), decarboxylated and exhaled as carbon dioxide (a minor pathway), or conjugated with glycine to form furoylglycine, which is excreted in urine (the major pathway).

B. Plant and Soil Metabolites of Furfural

Furfural degrades to 2-furoic acid and furfuryl alcohol in aerobic soil biodegradation environments. In anaerobic environments, ring opened metabolites propionic acid and acetic acid are also observed. Upon aqueous photolysis, several major ring opened products are formed; they include succinic acid, malonic acid, 2-ketoglutaric acid, propionic acid and formic acid. Some of these ring opened products are part of the Kreb's cycle (*e.g.* 2-ketoglutaric acid and succinic acid). It appears that, in soils, furfural metabolites may be of higher exposure concern than the parent due to furfural's lability.

2. Mutagenicity

A. Furfural

Furfural has been studied in a comprehensive battery of well-done genetic toxicology assays, many of which were sponsored by the National Toxicology Program (NTP) and are summarized below:

In Vitro Studies

Gene Mutation

As part of the NTP genetic toxicology screening of 270 chemical, furfural and furfuryl alcohol were tested by independent laboratories in the *Salmonella typhimurium* mammalian microsome mutagenicity assay using the standard plate incorporation and preincubation assays (Mortelmans *et al*., 1986). Both test material were negative in *S. typhimurium* TA100, TA1535, TA1537 or TA98 up to cytotoxic concentrations (furfural: ≥ 3333 µg/plate-S9 or + 10% rat or hamster S9) or the highest dose tested (furfural alcohol: 10,000 µg/plate-S9 or + 10% rat or hamster S9).

Furfural was also tested in *S. typhimurium* TA100, TA1535, TA1537, TA98 or TA102 for reverse gene mutations (MRID 46011017) at concentrations up to the limit dose for this test system (5000 µg/plate). Results were negative with or without S9 activation.

In another *Salmonella* mutagenicity assay, Marnett *et al*. (1985) found that furfural was not mutagenic in a preincubation assay with *S. typhimurium* strains TA102 or TA104 up to the maximum noncytotoxic concentration (1 µmole). These strains were used because they are more sensitive indicators of aldehyde and ketone mutagenicity than the standard Ames tester strain battery.

In contrast to the uniformly negative bacterial gene mutation assays, McGregor *et al.* (1988) demonstrated that furfural in the absence of S9 activation (an S9-activated assay was not performed), induced increases in the mutation frequency (MF) of L5178Y tk^+/tk^- mouse lymphoma cells. The response was dose-related (ranging from 1.6-fold increase at 100 µg/mL to 11.3-fold at 400 µg/mL), occurred at moderately cytotoxic concentrations (65- 11% relative total growth, RTG, respectively) and was confirmed in a repeat assay (2.3-fold increase in the MF with 27% RTG at 200 µg/mL). It is of note that when the global evaluation factor (GEF), which is a more recently accepted approach to evaluate mouse lymphoma data and is recommended by the international workshop on genotoxicity (Moore *et al*., 2002), was applied to these data, the conclusion remained positive.

Chromosome Aberrations

NTP also sponsored an *in vitro* mammalian cell chromosome aberration assay with furfural. In this study, Stich *et al.* (1981), exposed Chinese hamster ovary (CHO) cells to 0, 200, 300, 400 or 500 µg/mL -S9 for 8-10 hours or 0, 500, 760, 1000, or 1230 µg/mL +S9 for 2 hours. Due to marked cell cycle delay, cultures were harvested at 22- 23.5 hours after treatment. Metaphase analysis revealed that in the absence, but not presence, of S9-activation, the percentage of cells with chromosome aberrations was pair-wise significantly ($p<0.05$) increased at 400 and 500 µg/mL-S9 with a significant ($p<0.001$) trend.

Other Mutagenic Mechanisms

As part of the comprehensive investigation of the toxicology of furfural, NTP sponsored an *in vitro* investigation of sister chromatid exchanges (SCEs) in CHO cells. Accordingly, Stich *et al*. (1981), exposed CHO cells to 0, 11.7, 38.9 or 117 µg/mL -S9 for 8-10 hours or 0, 117, 389, or 1170 µg/mL +S9 for 2 hours. Cells were processed and second division metaphases were analyzed. All non-activated or S9-activated concentrations induced significant pairwise ($p<0.01$) and dose-related increases (trend: $p<0.001$) in the percentage SCEs/chromosomes. The response was appreciably stronger in the absence of metabolic activation.

The ability of furfural and furfuryl alcohol to induce SCEs in mammalian cells was also assayed by Gomez-Arroyo and Souza (1985). In this study, human lymphocytes, collected from healthy donors, were dosed with 3.5-14.0 x 10^{-5} M furfural or 3.3-9.9 x 10^{-3} M furfuryl alcohol for 70 hours; 50 metaphases per duplicate culture were examined and SCE frequencies were determined. Results indicated that furfural at 7.0 and 14.0 x 10^{-5} M induced significant ($p<0.001$) and dose-related increases in SCEs. By contrast, furfuryl alcohol was negative. The effect of comparable concentrations of furfural on the mitotic spindle in the human lymphocytes from healthy donors was further evaluated. Data for the 24- and 48-hour harvest intervals show significant ($p<0.001$) and dose-related increases in c-mitosis, indicative of a mitotic poison along with significant ($p<0.05$-0.001) and dose-related increased mitotic indices, indicative of stimulated cell division. The percentage of tetraploid cells was also increased significantly ($p<0.05$) at 14 x 10^{-5} M furfural but only at the 48-hour cell harvest.

Additional blood samples were collected from 6 workers occupationally exposed to furfural or furfuryl alcohol. The analysis of SCE in these workers showed no significant differences compared to the control group of 6 workers without exposure.

In Vivo Studies

In a *Drosophila melangaster* sex -linked recessive lethal mutation assay, Woodruff *et a*l. (1985) exposed 24-hour old Canton-S males to furfural either by abdominal injection (100 ppm, 24-hour recovery) or feeding (1000 ppm, 3 days). Treated males were mated with three *Basc* females for 3 days and remated with fresh females to produce three broods which sampled sperm over the entire period of spermatogenesis. A significant ($p<0.05$) increase in sex-linked recessive lethal mutations was observed in the male germ cells after injection of 100 ppm furfural. No increases were seen in the feeding phase of study. In a follow-up experiment, the same investigators found that the administration of 100 ppm via injection did not induce reciprocal translocations in *D. melanogaster* males.

In the NTP *in vivo* mouse bone marrow cytogenetic assays, furfural was neither clastogenic nor induced SCEs in the bone marrow cells of male B6C3F1 mice administered doses of 0, 50, 100 or 200 mg/kg by intrapertoneal injection.

From these data it can be concluded that there is evidence of gene mutations *in vitro* in mammalian cells (mouse lymphoma L518Y) but not in bacteria. Similarly, there is convincing evidence of chromosome aberrations (CHO cells) and SCE induction (human lymphocytes and CHO cells) *in vitro* but this genotoxic activity is not expressed *in vivo* in mouse bone marrow cells. Sex-linked recessive lethal mutations in *D. melangaster* male germinal cells were also seen following abdominal injection but not when furfural was administered via feeding for 3 days. Based on the few studies reporting evidence of gene mutations, the European Union rapporteur of the risk assessment for furfural recommended that an *in vivo* gene mutation assay should be performed to elucidate the biological relevance of the genetic toxicology results.

Accordingly, an *in vivo* gene mutation assay with λlacZ-transgenic male mice (MRID 46011018) was submitted to the Agency. In this study, five groups of 15 male transgenic mice with *lac*Z genes as the mutational target received furfural prepared in corn oil at 0, 37.5, 75, 150 or 300 mg/kg/day by oral gavage for 28 days. On Day 28, 3 mice/group were sacrificed and selected tissues (*i.e.,* liver) were collected and examined histologically. On Days 62 and 63, the remaining animals were sacrificed and genomic DNA was harvested from the liver. The *lac*Z genes were packaged in lambda phages, mixed with *Escherichia coli* C *lac*Z*rec*A*gal*E, plated and the mutation frequencies (MFs) were determined.

Dose selection was based on the findings of a 13-week subchronic toxicity study with a NOAEL of 75 mg/kg/day; 300 mg/kg/day was selected as the high dose which was expected to induce hepatotoxicity (Irwin, 1990). In the mutational assay, furfural was tested up to a toxic dose (300 mg/kg/day) based on three unscheduled deaths (3 of 10 animals), significant increases in both the absolute and relative liver weights accompanied by findings of slight centrilobular hypertrophy (3 of 3 mice), focal hemorrhage and inflammatory response (1of 3), and focal aggregates of mononuclear cells (1of 3) after 28 days of treatment. Despite the evidence of hepatotoxicity, there was no indication of a mutagenic response in the livers harvested from mice exposed for 28 days and allowed a 34 or 35 day treatment-free expression time. Based on these considerations, furfural did not induce an *in vivo* mutagenic response in this transgenic mouse test system.

Overall Conclusions for Furfural

Furfural has been studied in a comprehensive battery of acceptable genetic toxicology assays, many of which were sponsored by the NTP, provided valuable information, and are acceptable for regulatory purposes. Furfural is uniformly negative in bacterial assays for gene mutations but is mutagenic in cultured mammalian cells (mouse lymphoma). It is also clastogenic and induces SCEs in cultured CHO cells as well human lymphocytes. It is of note that the genotoxic response is more apparent in the absence rather than the presence of exogenous rodent metabolic activation. This observation is consistent with the negative findings from assays in whole animals regardless of the genetic endpoint examined (e.g., chromosome aberrations and SCEs in mouse bone marrow or gene mutations in transgenic mice) as well as the negative SCE results found in worker occupationally exposed to furfural. Overall, the data suggest that while furfural has intrinsic mutagenic potential in cultured mammalian cells, it is not expressed in whole animals since it is rapidly metabolized by the liver and rendered either non-mutagenic or markedly less mutagenic. Additionally, the negative data for the *in vivo* gene mutations assay, which examined the mouse liver as the target for furfural-induced tumorigenic activity, rule out mutagenicity as a possible mode of action for the induction of liver tumors seen in the 2-year mouse bioassay. Based on these considerations, there is no concern for mutagenicity.

B. Furfuryl Alcohol

The hydrogenated product of high pressure reduction of furfural, furfuryl alcohol was also assayed in a series of genetic toxicology studies sponsored by the NTP; summaries are presented below:

In Vitro Studies

Gene Mutation

The negative results of the NTP-sponsored *S. typhimurium* mammalian microsome mutagenicity assay conducted by Mortelmans *et al*. (1986) are discussed above with furfural.

Monien et al., (2011) found furfuryl alcohol to be mutagenic in *Salmonella typhimurium* TA100 strains engineered to express human sulfotransferase (TA100 SULT1A1*1 or TA100 SULT1A1*1Y), which is similar to the cytosolic protein level found in human liver (i.e., 0.3% or 2.6%, respectively). Additionally, the investigators found evidence of two DNA adducts [N^2-((furan-2-yl) methyl)-2' –deoxyguanosine (N^2-MFdG) and N^6-((furan-2-yl) methyl)-2' –deoxyadenosine (N^6-MFdA) in the bacterial DNA. Increases in revertant colonies were 2- and 4-fold higher than background for the strain showing the lower expression (TA100 SULT1A1*1) and 4- and 7-fold for the strain with the higher expression (TA100 SULT1A1*1Y) at 25 or 100 nmol/plate furfuryl alcohol, respectively. The investigators further reported that these findings correlated with the occurances of these adducts in the liver, lungs, and kidney of FVB/N mice receiving ≈390 mg/kg/day of furfuryl alcohol in drinking water for 28 days.

The findings of these investigation should be viewed with caution for several reasons: 1) there is little or no information on the bacterial strain characteristics, (*e.g.,* no historical control data); 2) the data should be confirmed in an independent study; 3) no primary data were presented; therefore, the presentation of means and standard errors instead of standard deviations for the bacterial mean values does not allow an independent assessment of variation around the means, 4) no primary data were presented for DNA adducts in the liver , kidney or lung of male mice, 5) findings of N^2-MFdG or N^6-MFdA adducts did not correlate with the induction of tumors at specific sites in the NTP mouse bioassay (e.g., similar levels of adducts were reported in the mouse liver, lung and kidney; however, the only site of tumor formation in the lifetime inhalation bioassay mouse bioassay was the renal tubules. Furthermore, the dose at which adducts were detected was higher than the established maximum tolerated dose (60 mg/kg/day) and the tumorigenic dose for the NTP study. Based on these considerations, CARC concluded that these data do not provide reliable evidence of an *in vivo* genotoxic response.

Chromosome Aberrations

NTP also sponsored an *in vitro* mammalian cell chromosome aberration assay with furfuryl alcohol. In this study, Stich *et al.* (1981), exposed Chinese hamster ovary (CHO) cells to 0, 160, 300 or 500 µg/mL -S9 for 10 hours or 0, 300, 500 or 1000 µg/mL +S9 for 2 hours. Cultures were harvested at 12- 13 hours after treatment. Metaphase analysis revealed that in the absence of S9 activation, furfuryl alcohol was not clastogenic. With S9, the percentage of cells with chromosome aberrations was significantly ($p<0.05$) increased at 500 and 1000 µg/mL but the increase was not dose related or reproduced in a repeat trial.

Other Mutagenic Mechanisms

An *in vitro* investigation of sister chromatid exchanges (SCEs) in CHO cells was sponsored NTP and was conducted by Stich *et al*. (1981). In this study, CHO cells were exposed to 0, 16, 50, 160 or 500 µg/mL -S9 for 26 hours (Trial 1) or comparable non-activated concentrations for 26 hours (Trial 2) or 0, 16, 50, 160 or 500 µg/mL +S9 for 2 hours. Cells were processed and second division metaphases were analyzed. The non-activated test material at 500 µg/mL induced a positive increase in the percentage SCEs/chromosomes in Trial 1. This result was confirmed in Trial 2 with positive effects at 160 and 500 µg/mL -S9. By contrast, the S9-activated test material was not genotoxic.

The negative findings of the SCE study of Gomez-Arroyo and Souza (1985), with cultured human lymphocytes or lymphocytes collected from workers occupationally exposed to furfural or furfuryl alcohol, were previously discussed with furfural.

In Vivo Studies

In the NTP *in vivo* mouse bone marrow cytogenetic assays, furfuryl alcohol was not clastogenic in the bone marrow cells of male B6C3F1 mice harvested 17 hours after administration of 0, 75, 150 or 300 mg/kg by intrapertoneal injection. In bone marrow cells harvested at 36 hours after exposure to 0, 50, 100 or 200 mg/kg, no significant pairwise increases were observed but a significant ($p<0.05$) trend in cells with chromosome aberrations was seen. However, these results were not reproduced in two subsequent independent trials using comparable test material doses and a comparable harvest time. It was, therefore, concluded that furfuryl alcohol was not clastogenic in whole animals. A similar conclusion was drawn for the mouse bone marrow micronucleus assay performed with 6 groups of 5 male B6C3F1 mice dosed intraperitoneally with 0, 15.625, 31.25, 62.5, 125, or 250 mg/kg furfuryl alcohol/day for 3 consecutive days. Finally, furfuryl alcohol did not induce an increase in SCE in mouse bone marrow cells of male B6C3F1 mice receiving 0, 75, 150 or 300 mg/kg and sacrificed at 23 hours post-treatment or at 0, 37.5, 75 or 150 mg/kg and harvested at 23 hours post-treatment.

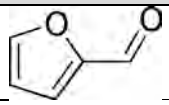
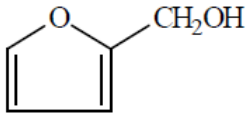
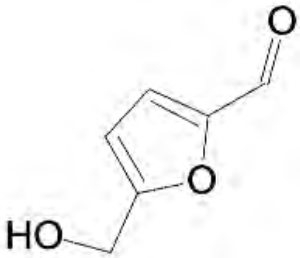
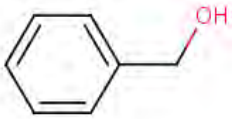
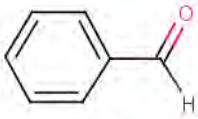
Overall Conclusions for Furfuryl Alcohol

Furfuryl alcohol has been studied in a battery of acceptable genetic toxicology assays, many of which were sponsored by the NTP, provided valuable information, and are acceptable for regulatory purposes. The data indicate that furfuryl alcohol is not mutagenic in bacteria and does not cause chromosome aberrations or SCE induction in mammalian cells. These *in vitro* data are supported by the results of whole animals studies showing that furfuryl alcohol was not clastogenic, aneugenic or genotoxic in mouse bone marrow cytogenetic, micronucleus or SCE assays. It is, therefore, concluded that furfuryl alcohol does not present a mutagenic concern.

3. Structure-Activity Relationship

The available literature was screened for relevant information on structural analogs of furfural and furfuryl alcohol. The chemical name, CAS registry number and structure of the analogs identified are listed in Table 25. A brief discussion of the mutagenicity and carcinogenicity study results from NTP are provided.

Table 25. Furfural, furfuryl alcohol and their structural analogs.

Chemical Name	CASRN	Structure
Furfural	98-01-1	
Furfuryl alcohol	98-00-0	
Furan	110-00-9	
5-(Hydroxymethyl)-2-furfural	67-47-0	
Benzyl Alcohol	100-51-6	
Benzaldehyde	100-52-7	

Furan was negative in the Ames assay and did not induce unscheduled DNA synthesis in rat or mouse hepatocytes following treatment *in vitro* or *in vivo*. Furan did induce gene mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells in the absence of metabolic activation and was observed to induce chromosomal aberrations at 250 mg/kg bw in the bone-marrow cells of B6C3F1 male mice injected intraperitoneally, when an extended protocol involving a late harvest time was employed. **Furan is classified as a Group 2B carcinogen (possibly carcinogenic to humans) by IARC (IARC, vol 63). There is sufficient evidence of carcinogenicity in animals with cholangiocarcinomas observed in the liver and mononuclear cell leukemia (NTP, 1993).**

5-(Hydroxymethyl)-2-furfural (5-HMF) was weakly mutagenic in *S. typhimurium* strain TA 100 in the absence of exogenous metabolic activation in one study. In the second study by the NTP, no mutagenicity was detected with or without activation in any strain. In an oral gavage chronic/carcinogenicity study of 5-(hydroxymethyl)-2-furfural by NTP, the NTP concluded that there was no evidence of carcinogenic activity in male or female rats or in male $B6C3F_1$ mice. **There was some evidence of carcinogenic activity for 5-HMFin $B6C3F_1$ female mice based on increased incidences of hepatocellular adenomas (EFSA, 2011).**

Benzyl Alcohol was not mutagenic in *S. typhimurium* in the presence or absence of exogenous metabolic activation. In the mouse L5178Y/TK+/- lymphoma assay, benzyl alcohol induced an increase in triflurothymidine (Tft)-resistant cells in the absence, but not in the presence, of S9; the effect was associated with toxicity. With Chinese hamster ovary (CHO) cells, an equivocal increase in sister chromatid exchanges both with and without S9 and a significant increase in chromosomal aberrations in the presence of S9 (but not the absence) was observed. In an oral gavage chronic/carcinogenicity study of benzyl alcohol conducted by NTP there was no evidence of carcinogenic activity in male or female F344/N rats or in male or female $B6C3F_1$ mice.

Benzaldehyde was not mutagenic in *S. typhimurium* and did not induce chromosomal aberrations in CHO cells, with or without exogenous metabolic activation. Benzaldehyde induced increases in triflurothymidine-resistant mouse lymphoma cells in the absence of exogenous metabolic activation and increased sister chromatid exchanges in CHO cells in both the presence and absence of metabolic activation. In an oral gavage chronic/carcinogenicity study of benzaldehyde conducted by NTP, there was no evidence of carcinogenic activity in male or female F344/N rats at doses up to 400 mg/kg per day and **there was some evidence of carcinogenic activity in male or female $B6C3F_1$ mice, as indicated by increased incidences of squamous cell papillomas and hyperplasia of the forestomach.**

Being a direct-acting reactive electrophilic chemical, furfural is expected to have a greater potential for inducing cancer by the inhalation route than the oral route because of the portal-of-entry effect. A structurally related chemical, formaldehyde, is a well known carcinogen via the inhalation route but does not seem to be a serious cancer concern by the oral route unless there is a massive exposure to overwhelm the detoxification capacity. There is some suggestive evidence that the nasal carcinogenic effect of furfuryl alcohol via the inhalation route may be related to its oxidation to furfural as the proximate or ultimate carcinogen.

4. Subchronic and Chronic Toxicity

The primary target organ for furfural exposure via the oral route is the liver while the primary target organ for exposure via the inhalation route is the nasal cavity. In two independent studies conducted under the U.S. National Toxicology Program (NTP), Furfural (99% a.i.; Lot # Q112979) was administered for up to 13 weeks in corn oil via gavage to 10 F344/N rats/sex/group at nominal dose levels of 0, 11, 22, 45, 90 or 180 mg/kg/day or 10 $B6C3F_1$ mice/sex/group at 0, 75, 150, 300, 600, or 1200 mg/kg/day. The dosages were administered daily 5 days/week at dose volumes of 5 mL/kg in the rats and 10 mL/kg in the mice. Survival, body weight, body weight gain, and organ weight data were provided. Histopathology liver findings were summarized in the text (MRID 46011015).

In the rat study, 9/10 males and 10/10 females in the 180 mg/kg group, and 1/10 males and 4/10 females in the 90 mg/kg group died before the end of the study. The majority of the 90 mg/kg deaths were due to gavage injury. Mean body weights and body weight gains were similar to controls; terminal body weights were only slightly increased ($p \leq 0.05$) in the 45 and 90 mg/kg males compared to controls. In the 90 mg/kg male rats, increases (p less than or equal to 0.05) in absolute and relative (to body) liver weights were observed. A non-dose dependent increase in the incidence of minimal to mild hepatocyte cytoplasmic vacuolization was observed in controls and all treated males (4/10, 10/10, 10/10, 10/10 and 9/10 at 0, 11, 22, 45, and 90 mg/kg, respectively). Based on this study, the NTP selected 60 mg/kg/day as the high dose and 30 mg/kg/day as the low dose for the subsequent two year rat study. The Systemic Toxicity NOAEL is 45 mg/kg/day and the Systemic Toxicity LOAEL is 90 mg/kg/day based on liver weight changes and liver pathological observations. The observation data available in this study for endpoint determination was minimal; this study was used as a range-finding study for the NTP carcinogenesis study.

In the mouse study, all animals that received 1200 mg/kg and the majority of the 600 mg/kg group died within the first few weeks of the study. These deaths were considered treatment-related. At 150 and 300 mg/kg, mean body weights, body weight gains, and terminal body weights were slightly decreased in the males and were similar to controls in the females. Increased (p less than or equal to 0.05) relative (to body) liver weights were observed in the 300 mg/kg males and the 75, 150, and 300 mg/kg females. It was stated that centrilobular hepatocyte coagulative necrosis was observed in the 1200 mg/kg group (8/10 males and 2/10 females) and in males at 600 mg/kg (9/10), 300 mg/kg (1/10), and 150 mg/kg (1/10). Inflammation, characterized by a minimal to mild mononuclear inflammatory cell infiltrate, was also observed in the presence of liver necrosis. Based on this study, the NTP selected 175 mg/kg/day as the high dose and 50 mg/kg/day as the low dose for the subsequent mouse carcinogenicity study. The Systemic Toxicity NOAEL is less than 75 mg/kg/day and the Systemic Toxicity LOAEL is equal to or greater than 75 mg/kg/day based on liver weight changes and liver pathological observations. The observation data available in this study for endpoint determination were minimal. This study was used as a range-finding study for the NTP carcinogenesis study.

In a subchronic inhalation toxicity study (MRID#'s 46426504 and 46426505), furfural (99% a.i.) commercially obtained from Sigma/Aldrich, Brussels, was administered as a vapor by the nose-only inhalation route to 5 rats/sex/group (Fischer 344 strain) initially to concentrations 0, 40, 80, 160, 320, 640, and 1280 for 6 hours per day, 5 days per week for 28 weeks. These groups were designated as Groups A to G, respectively. Additional treatment groups exposed to periods of 3 hours/day (5/sex/group) were exposed to furfural vapors at 320, 640 and 1280 mg/m^3, 5 days per week for 28 days. These groups were designated as H, I and J, respectively. Because of excessive mortalities in groups F, G and J, this design was changed. Group F (640 mg/m^3) was discontinued and two new groups with fresh animals were set up: 20 mg/m^3.for 6 hour exposures, designated herein as G2; 160 mg/m^3 for 3 hour exposure periods, designated as J2.

The inhalation treatment groups were evaluated daily for toxicity, weekly for body weight and food consumption, terminally for hematology changes, clinical chemistry and gross and histopathological effects. Group F (640 mg/m^3) was dropped after deaths occurred during day 1 and day 8. All animals exposed to concentrations of 1280 mg/m^3 whether for 6 hours (Group G) or for 3 hours, Group J, died in the first day of exposure. These groups were reconstituted at lower concentrations and designated G2 and J2 as noted above. There was no additional mortality in the revised dosing treatments for the rest of the study.

Body weight, food consumption, and clinical pathology were not adversely affected by the inhalation treatments. Pathological changes were seen in the nasal epithelium, some seen effecting all animals at all treatment levels. Other effects were generally dose related. Treatment related pathological effects were limited to olfactory and respiratory epithelium of the nasal cavity. There were no treatment related effects on the kidney, liver, spleen and thymus pathology. Respiratory epithelial atypical hyperplasia was seen in all treated males and females (5/5) for 6 hour exposure groups 20 mg/m^3 to 320 mg/m^3 (Groups G2, B, C, D, and E) and 3 hour exposure groups of 160 mg/m^3 to 640 mg/m^3 (Groups J, H and I). Respiratory epithelial squamous metaplasia was also found in all males and female (5/5) for the same 6 hour exposure groups (G2, B, C, D and E) and all of the females (5/5) for the 3 hour exposure groups (J2, H and I) and 3-4/5 males in the same 3 hour exposure groups. Respiratory epithelial squamous metaplasia and atypical hyperplasia were seen in males and females in a suggestive dose-response from the lowest concentration to the higher ones. Thus there were no inhalation treatments that did not result in nasal epithelium damage; however, the severity of the damage was noted to be less intense in the 3 hour exposure groups compared to the 6 hour exposure groups of animals. The Systemic Toxicity LOAEL is 20 mg/m^3 (the lowest dose tested) based on nasal epithelial pathology seen throughout all of the treated animal groups. There was no Systemic Toxicity NOAEL established.

5. Mode of Action Studies

Data to support a mode of action (MOA) analysis for the liver tumors in furfural treated animals and the liver and nasal tumors in furfuryl alcohol treated animals were not provided.

V. COMMITTEE'S ASSESSMENT OF THE WEIGHT-OF-THE EVIDENCE

Furfural

Furfural in corn oil was administered by gavage to F344/N rats (50/sex/dose) at dose levels of 0, 30, or 60 mg/kg bw/day, 5 days/week for 2 years (104 weeks). Furfural in corn oil was administered by gavage to B6C3F1 mice (50/sex/dose) at dose levels of 0, 50, 100, or 175 mg/kg bw/day, 5 days/week for 2 years (104 weeks).

The CARC considered the following for a weight-of-evidence determination of the carcinogenic potential of furfural.

Evidence for Carcinogenicity

Rat

Cholangiocarcinomas of the liver: Two male rats at the high dose group (60 mg/kg/day) had this rare tumor. The incidence (4%), although not statistically significant, was outside of the historical control level (0.4% ± 0.88%) for this tumor type. In addition, these lesions were corroborated by the presence of non-neoplastic lesions (bile duct dysplasia with fibrosis and centrilobular necrosis) in male rats at the high dose. Bile duct dysplasia with fibrosis is considered to be an early stage in the development of cholangio-carcinomas. The concern for the presence of this tumor was enhanced by the occurrence of this tumor (cholangiocarcinoma) in male rats exposed to Furan, a structurally related compound. **The CARC considered the cholangiocarcinomas of the liver in male rats to be treatment-related.**

Adequacy of Dosing: The doses tested were considered to be adequate, but not excessive, in both sexes to assess the carcinogenic potential of furfural. This was based on the results of the 16-day and 13-wee studies, and the presence of non-neoplastic histopathological liver lesions in males in this study.

Mouse

Liver Tumors: Male mice had statistically significant trends, and statistically significant pair-wise comparisons of the high dose (175 mg/kg/day) with the controls, for liver adenomas, carcinomas, and adenomas and carcinomas combined (all at $p < 0.01$). Female mice had significant trends for liver adenomas, and liver adenomas and/or carcinomas combined (at $p < 0.01$). There was a significant pair-wise comparison of the 175 mg/kg/day dose group with the controls for liver adenomas (at $p < 0.05$). All of these tumors are outside of the reported historical control incidence. These tumors were corroborated by the presence of non-neoplastic lesions (chronic inflammation and increased pigmentation of the liver) in both male and female mice. The concern for the presence of these tumors was enhanced by the occurrence of this liver tumor in $B6C3F_1$ female mice exposed to 5-methylflurfural and marked increases of hepatocellular

neoplasms in each sex of mice exposed to benzofuran, structurally related compounds. **The CARC considered the liver tumors in male and female mice to be treatment-related.** In males, there was also an increased incidence of kidney tumors in treated groups compared to controls; however, there were no statistically significant trends or pairwise comparisons for the tumor findings. **The CARC did not consider the kidney tumors in males to be treatment-related.** In females, there was a statistically significant trend for forestomach squamous cell papillomas, no statistically significant pairwise comparisons were observed. Although the incidence was outside of the historical control range, **the CARC did not consider the forestomach tumors to be of toxicological significance since the tumorscould be due to the irritating effect of gavage administration, none of the animals had malignant lesions of the forestomach and these tumors are not relevant for humans.**

Adequacy of Dosing: The highest dose tested was considered to be adequate, but not excessive, in both sexes, to assess the carcinogenic potential of furfural. This was based on the presence of non-neoplastic histopathological liver lesions (chronic inflammation and liver pigmentation) in males and females, and the results of the subchronic study.

Furfuryl Alcohol

Furfuryl alcohol was administered via inhalation to F344/N rats (50/sex/dose) at concentrations of 0, 2, 8, or 32 ppm for 6 hours/day, 5 days/week for 2 years (105 weeks). Furfuryl alcohol was administered via inhalation to B6C3F1 mice (50/sex/dose) at concentrations of 0, 2, 8, or 32 ppm, 5 days/week for 2 years (104 weeks)

The CARC considered the following for a weight-of-evidence determination of the carcinogenic potential of furfuryl alcohol.

Evidence for Carcinogenicity

Rat

Nasal tumors: Male rats had a statistically significant trend for nasal epithelial squamous cell carcinomas at $p<0.05$. There was also a significant trend at $p<0.01$, and a significant pair-wise comparison of the high dose group with the controls at $p<0.05$, for combined nasal lateral wall adenomas, epithelial adenomas, epithelial carcinomas and epithelial squamous cell carcinomas in male rats at the highest concentration (32 ppm). These tumors were corroborated by the presence of non-neoplastic lesions (hyperplasia of the nasal lateral wall, atrophy and metaplasia of the olfactory epithelium, and hyperplasia of the respiratory epithelium) which were observed in male and female rats in the 2-year study. These lesions increased in incidence and severity with increasing concentration. **The CARC considered the nasal tumors in male rats to be treatment-related. The CARC did not consider the nasal tumors and renal tumors observed in females to be treatment related due to the low incidences, lack of dose response relationship, absence of corroborative non-neoplastic lesions and lack of statistical significance.**

Adequacy of Dosing: The highest dose tested was considered to be adequate, but not excessive, in both sexes to assess the carcinogenic potential of furfuryl alcohol. This was based on the presence of non-neoplastic histopathological lesions in the nose (hyperplasia of the lateral wall, atrophy and metaplasia of the olfactory epithelium, and hyperplasia of the respiratory epithelium) in males and females, and the results of the subchronic study. Although there was 100% mortality at the high dose in the main study, the CARC did not consider this concentration to be excessive, since an adequate number of rats were available for data evaluation.

Mouse

Kidney Tumors: Male mice had statistically significant trends for renal tubule adenomas at $p < 0.05$. There was also a significant trend at $p < 0.01$, and a significant pair-wise comparison of the 32 ppm dose group with the controls at $p < 0.05$, for renal tubule adenomas and carcinomas combined. Ad hoc analyses were run on the male mouse kidney tumors because there were no individual animal data provided for the step sectioning performed on this tissue. These tumors were corroborated by the presence of non-neoplastic lesions (nephropathy and renal tubule degeneration) in both male and female mice in the cancer study. The severity of the lesions increased with increasing dose in male mice only. **The CARC considered the kidney tumors in male mice to be treatment-related.**

Adequacy of Dosing: The highest dose tested was considered to be adequate, but not excessive, in both sexes to assess the carcinogenic potential of furfuryl alcohol. This was based on the results of the 16-day and 13-week studies and the presence of non-neoplastic histopathological lesions in the kidney (nephropathy and renal tubule degeneration) in males in the cancer study.

VI. CLASSIFICATION OF CARCINOGENIC POTENTIAL

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005), the CARC classified **furfural as "Likely to Be Carcinogenic to Humans."** This determination was based on the following:

(i) Treatment-related cholangiocarcinoma of the liver, a rare tumor type, observed in male rats;
(ii) Treatment-related liver tumors (adenomas, carcinomas and/or combined adenomas/carcinomas) observed in male and female mice;
(iii) Occurrence of hepatocellular neoplasms in each sex of mice with compounds structurally very similar to furfural; and

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005), the CARC classified **furfuryl alcohol as "Likely to Be Carcinogenic to Humans."** This determination was based on the following:

(i) Treatment-related nasal tumors (adenomas, carcinomas and/or squamous cell carcinomas observed in male rats. ;
(ii) Treatment-related kidney tumors (adenomas, carcinomas and/or combined adenomas/carcinomas observed in male mice;

VII. QUANTIFICATION OF CARCINOGENIC POTENTIAL

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005), the CARC recommended quantification of human cancer risk using a linear approach since no mode of action data are available to support a non-linear mode of action for the tumor types seen with furfural and furfuryl alcohol.

VIII. CARC RECOMMENDATIONS

Based on the toxicological database, it appears that furfural may be more toxic via the inhalation route of exposure. Therefore, the CARC recommends that an inhalation carcinogenicity study be conducted for furfural.

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ATTACHMENT E:

Travlos, G.S., Hard, G.C., Betz, L.J., Kissling, G.E. (2011) Chronic progressive nephropathy in male F344 rats in 90-day toxicity studies: its occurrence and association with renal tubule tumors in subsequent 2-year bioassays. Toxicol Pathol. 39(2):381-389.

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Chronic Progressive Nephropathy in Male F344 Rats in 90-Day Toxicity Studies: Its Occurrence and Association with Renal Tubule Tumors in Subsequent 2-Year Bioassays

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Abstract

The occurrence and severity of spontaneous chronic progressive nephropathy (CPN) in control male F344 rats as well as the frequency of treatment-related CPN exacerbation were histopathologically reevaluated. A series of 43 National Toxicology Program (NTP) 90-day toxicity studies comparing the influence of NIH-07 or NTP-2000 diets was examined. Relationships between the histopathologic findings at 90 days and renal tubule proliferative lesions recorded in subsequent 2-year bioassays for 24 chemicals were statistically analyzed. CPN lesions were observed in 100% of the control male rats regardless of diet, but CPN was more severe in control rats fed NIH-07. Approximately one-third of the 90-day studies demonstrated a treatment-related exacerbation of CPN severity, which was independent of diet. For chemicals that proceeded to 2-year bioassays, all studies with a statistically significant increase in renal tubule tumors (RTT) at 2 years had treatment-related exacerbation of CPN in the 90-day and 2-year studies. These findings indicate that CPN occurs ubiquitously in young male F344 rats and that treatment-related exacerbation of CPN in 90-day studies is a relatively common occurrence, having the potential to be predictive of an increased incidence of RTT in subsequent 2-year bioassays.

Keywords: chronic progressive nephropathy; CPN; NIH-07 and NTP-2000 diets; toxicity and carcinogenicity studies; CPN exacerbation; renal tubule neoplasia.

Introduction

Chronic progressive nephropathy (CPN) is a common, spontaneous disease of the rat kidney, and it has been the subject of many detailed reviews (see Hard, Johnson, and Cohen 2009). While all strains of rat used in toxicity and carcinogenicity studies are affected, albino strains such as the F344 and Sprague-Dawley are more inclined to develop the disease. CPN occurs in both sexes but appears with greater frequency and severity in males. Although the etiology of CPN is unknown, numerous factors, mainly physiological, influence the incidence and severity of this spontaneous disease (Hard and Khan 2004). In addition to the male hormone effect (Baylis 1994), caloric restriction and dietary protein content are the most important factors in CPN, with caloric restriction being protective (Keenan et al. 2000) and high protein content increasing incidence and severity (Rao, Edmondson, and Elwell 1993). In 1994, in an effort to reduce various spontaneous lesions observed in toxicity and carcinogenicity studies, including CPN, the National Toxicology Program (NTP) replaced the NIH-07 diet, which contained ~24% protein, with the NTP-2000 diet containing ~14% protein.

The kidney is an important target organ in carcinogenicity bioassays conducted by the NTP, ranking along with liver as one of the most commonly affected sites for tumors in male rats (NTP 2010b), primarily tumors of the renal tubule (RTT). Of 498 NTP 2-year bioassays performed in male rats, 64 (12.9%) demonstrated site-specific tumor occurrence involving renal tubule cells (NTP 2010c, 2010b). However, in female rats, the kidney does not rank in the top 10 site-specific neoplasms, involving only 3.3% (18 of 504) bioassays (NTP 2010b). In contrast, RTT of spontaneous origin in male rats occurs with a relatively low incidence rate of 1.26% (44 of 3,484 animals) in NTP 2-year bioassays where NIH-07 diet was used (NTP 2010a). Female rats fed NIH-07 have an even lower incidence rate of 0.29% (10 of 3,474 animals; NTP 2010a). Based on the NTP historical control animal database, it appears that dietary manipulations designed to mitigate CPN and its potential long-term effects also lowered the incidence of spontaneous RTT. NTP 2-year bioassays using the NTP-2000 diet have continued to demonstrate a gender difference for the occurrence of spontaneous RTT, with the incidence rate decreasing to 0.62% (8 of 1,294 animals) and 0.16% (2 of 1,236 animals) for male and female F344 rats, respectively (NTP 2010a). Along with the alpha$_{2u}$-globulin (α_{2u}-g) nephropathy mode of action (Hard et al. 1993; Swenberg and Lehman-McKeeman 1999), a potential explanation for the predilection of the kidney as a common site for chemically induced neoplasms in male rats and the gender difference in RTT incidence might be chemical exacerbation of CPN (Hard, Johnson, and Cohen 2009).

Since doses used in NTP 2-year bioassays are selected based on data obtained from 90-day subchronic toxicity studies, renal

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Abbreviations: α_{2u}-g, alpha$_{2u}$-globulin; CPN, chronic progressive nephropathy; H&E, hematoxylin and eosin; NTP, National Toxicology Program; RTT, renal tubule tumor.

TABLE 1.—Ninety-day NTP toxicity studies (along with diet and route of administration) in which kidney histopathology was reevaluated.[a]

Chemical Name (NIH-07 Diet)	Route	Chemical Name (NTP-2000 Diet)	Route
Coconut oil diethanolamine (TR 479)	Dermal	Pentaerythritol triacrylate (GMM 4)	Dermal
Dicyclohexylcarbodiimide (GMM 9)	Dermal	Trimethylolpropane triacrylate (GMM 3)	Dermal
Diisopropylcarbodiimide (TR 523)	Dermal	p-tertiary-butylcatechol (TS 70)	Feed
Lauric acid diethanolamide (TR 480)	Dermal	Chromium picolinate monohydrate (TR 556)	Feed
Oleic acid diethanolamide (TR 481)	Dermal	Citral (TR 505)	Feed
Anthraquinone (TR 494)	Feed	Transcinnamaldehyde (TR 514)	Feed
Benzophenone (TS 61, TR 533)	Feed	Acrolein (TS 48)	Gavage
p,p-dichlorodiphenyl sulfone (TR 501)	Feed	Allyl alcohol (TS 48)	Gavage
trans-1,2-dichloroethylene (TS 55)	Feed	Butanal oxime (TS 69)	Gavage
1,1,2,2-tetrachloroethane (TS 49)	Feed	2,4-decadienal (TS 76)	Gavage
o-chloroaniline (TS 43)	Gavage	Elmiron (TR 512)	Gavage
Oxymetholone (TR 485)	Gavage	Estragole (TS 82)	Gavage
3,3,4,4-tetrachloroazobenzene (TS 65)	Gavage	Formamide (TR 541)	Gavage
3,3,4,4-tetrachloroazoxybenzene (TS 66)	Gavage	2,4-hexadienal (TR 509)	Gavage
Methylene blue trihydrate (TR 540)	Gavage	Alpha-methyl styrene (TR 543)	Inhalation
2-butoxyethanol (TR 484)	Inhalation	Decalin (TR 513)	Inhalation
Indium phosphide (TR 499)	Inhalation	Divinylbenzene (TR 534)	Inhalation
Isobutene (TR 487)	Inhalation	Propylene glycol mono-t-butyl ether (TR 515)	Inhalation
1-nitropyrene (TS 34)	Inhalation	Stoddard solvent IIC (TR 519)	Inhalation
Benzyltrimethylammonium chloride (TS 57)	Water	Dibromoacetic acid (TR 537)	Water
Urethane/water (TS 52)	Water	Sodium dichromate dihydrate (TS 72, TR 546)	Water
Urethane/alcohol (TS 52)	Water		

[a] For the chemicals listed, the number in parentheses is the National Toxicology Program (NTP) report number; TS = NTP Toxicity Report. Series, TR = NTP Technical Report Series, GMM = NTP Genetically Modified Model Report Series.

histopathology in subchronic studies might provide insight for kidney lesions occurring in the chronic studies. The present study was therefore designed to (1) assess the frequency of CPN occurring in control F344 male rats in 90-day studies, (2) determine the frequency of chemical exacerbation of CPN occurring in 90-day studies, and (3) examine possible relationships between treatment-induced exacerbation of CPN at 90 days and microscopic findings (including occurrence of RTT) at 2 years. Accordingly, histopathologic reevaluation of CPN and other kidney lesions in control and treated male F344 rats was conducted for a series of NTP 90-day studies. The findings from this assessment were then compared with the 2-year outcomes recorded for those chemicals that proceeded to 2-year bioassays. Since diet can have a profound biological effect on CPN, the effects of the two standardized, open formula diets, NIH-07 and NTP-2000, were compared.

MATERIALS AND METHODS

Study Evaluation

Kidneys from forty-three 90-day toxicity studies (Table 1) conducted by the NTP including control and highest-exposure treated male F344 animals were reevaluated for incidence and severity of CPN and incidence of non-CPN lesions (1,090 rats in total). Since the NTP had changed to the NTP-2000 diet, which contained less protein and more fiber and fat than the original NIH-07 diet, 90-day studies were selected as close to the time of the diet switch in 1994 as possible; all were conducted from 1991 to 2001. Of the 43 studies, 22 used the NIH-07 diet and 21 the NTP-2000 diet. Both diets were produced and supplied by Zeigler Brothers, Inc. (Gardners, PA).

For chemicals with a 2-year carcinogenicity bioassay, CPN and non-CPN kidney lesions were also reevaluated in the 90-day exposure groups that most closely approximated the highest-exposure group used in the 2-year study (2-year–equivalent dose). The 90-day kidney lesions were compared with the 2-year histopathologic findings recorded in the NTP Technical Reports. For six of the chemicals (diisopropylcarbodiimide, isobutene, chromium picolinate, decalin, divinylbenzene and propylene glycol monobutyl ether), the 2-year–equivalent dose was also the highest-exposure dose in the 90-day study.

The studies had been conducted in accordance with the standard protocols established by the NTP (Chhabra et al. 1990). In general, for the 90-day studies, groups of 10 male and 10 female F344 rats (Taconic Laboratory Animals and Services, Germantown, NY) had been either untreated or exposed to the vehicle used (control groups) or exposed to one of five doses of a chemical for 13 consecutive weeks. All rats were approximately 4 to 7 weeks old at study commencement. Kidneys (along with other tissues for histopathology) had been fixed in 10% neutral-buffered formalin, and 5-μm sections were stained with hematoxylin and eosin (H&E). For the 2-year carcinogenicity bioassays, groups of 50 male and 50 female F344 rats had been untreated or exposed to a vehicle (controls) or exposed to one of two or three dose levels of chemical for 104 consecutive weeks. Histopathological data for kidneys from the 2-year bioassays were taken directly from the NTP Technical Reports, and kidney sections from those studies were not reevaluated in this investigation.

Assessment of kidney histopathology in the 90-day studies was performed by one of the authors (G.C.H.). H&E sections of both kidneys (one transverse, one sagittal in most cases) from all control and high-exposure male animals of the forty-three 90-day studies were examined in their entirety in an up-and-down battlement pattern for evidence of CPN or treatment-related lesions. Male rats were chosen for review because they are more affected by CPN than female rats (Gray 1977). For chemicals tested in 2-year carcinogenicity bioassays, kidney sections from the 90-day-dose groups that approximated (identical or closest to) the highest dose used in the 2-year study were examined in the same manner.

Grading of CPN

A specialized system for grading CPN was used, based on in-depth study of the pathogenesis of this spontaneous disease over many years (Hard and Khan 2004; Hard, Johnson, and Cohen 2009). The earliest stage of CPN was identified as single basophilic tubules with thickened basement membranes (Figure 1a) usually in the cortex, foci of tubules with the same characteristics, or tubules dilated with a hyaline proteinaceous cast in the medulla (Figure 1b). The severity of CPN was graded on a scale ranging from 0 to 8 (Hard and Khan 2004). In this system, 0 represented *no lesions* observed in either kidney section, 1 = *minimal* ($\leq$5 lesions per both sections), 2 = *mild* (6–15 lesions), 3 = *low-moderate* (16–30 lesions), 4 = *mid-moderate* (30–60 lesions), 5 = *high-moderate* (focal lesions/casts too numerous to count), 6 = *low-severe,* 7 = *high-severe,* 8 = *end-stage.* Grades 1 through 5 represented increasing numbers of focal lesions. Grade 6 represented a stage at which the focal lesions began to coalesce into areas. In grade 7, the majority of the cortical parenchyma was affected, while in grade 8, little or no normal parenchyma remained, representing an end-stage kidney.

Statistical Analyses

The Wilcoxon rank-sum test (Hollander and Wolfe 1973) was used to test differences in effect between the two diets on CPN severity in 90-day control groups; the data were also analyzed separately for each route. The Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison's procedure (Hollander and Wolfe 1973) was used to determine whether routes of chemical exposure were statistically different for CPN severity in the control groups; the data were analyzed separately for each diet. The association of CPN severity with other endpoints of renal change (e.g., absolute and relative kidney weights) was determined using Spearman's rank correlation coefficient (Hollander and Wolfe 1973) on the mean values reported for each study; control and high-dose groups were analyzed separately. A two-sided, 0.05 level of significance was used for these analyses.

To assess the effects of chemical treatment on CPN, the mean severity grade for the control, high-, and 2-year–equivalent dose groups were calculated for each chemical. The difference between the control and high-dose group or the control and 2-year–equivalent means was found. A difference of $\geq$0.5 in severity was considered to be an increase (i.e., exacerbation) in CPN, while a difference of $\leq$–0.5 in severity was considered a decrease (i.e., reduction); values between –0.5 and 0.5 were considered no change. While this was an arbitrary decision point, the cutoff was chosen based on experience and used to reconcile statistically significant differences that would otherwise be overlooked. Jonckheere's trend (Hollander and Wolfe 1973) and Wilcoxon's tests supported the conclusions based on this cutoff. An exact chi-square test (Hollander and Wolfe 1973) was performed to compare trend calls (i.e., exacerbated, reduced, or no change in CPN) between diets and route of exposure. It was also used for comparing CPN trend calls in the 2-year–equivalent dose of the 90-day studies with the neoplastic and nonneoplastic findings reported in the NTP 2-year studies.

Kidney tumor incidences from the NTP 2-year studies were reanalyzed for this investigation using the poly-3 test (Bailer and Portier 1988) and a hybrid test that incorporated historical control data (Peddada, Dinse, and Kissling 2007); kidney hyperplasia incidences were analyzed using the poly-3 test. Incidences of RTT or hyperplasias were considered increased if they were significantly elevated above the control group in at least one dose group or if there was a significant increasing trend with dose at one-sided $p \leq 0.05$. In keeping with NTP practices, historical control groups were comprised of data from control animals in studies using the same route of exposure and conducted within the most recent 5 years prior to the study of interest. The poly-3 test has been in use for NTP 2-year studies since 1999. The hybrid statistics are now being routinely calculated on all new NTP 2-year studies and will be included in NTP technical reports subject to acceptance by the NTP Board of Scientific Counselors.

Results

Studies Evaluated

The chemicals evaluated in this review of 90-day studies are listed in Table 1 according to diet type and route of exposure. There were approximately equal numbers of studies for the two diets (22 for NIH-07 and 21 for NTP-2000), but the distribution of route of exposure varied between diets. For example, the NIH-07 diet had a relatively equal number of studies for the different routes of exposure (n = 5, 5, 5, 4, and 3 for dermal, feed, gavage, inhalation, and drinking water routes of exposure, respectively). In contrast, the NTP-2000 diet had a higher number of gavage studies (8) and a lower number of dermal studies (2) compared with the NIH-07 diet.

CPN in Control Male Rats

Regardless of diet or route of exposure, early lesions of CPN were found in 100% of control male rats in all of the forty-three 90-day studies (n = 220 and 230 animals for NIH-07 and NTP-2000, respectively). Severity grades ranged from 1 to 4, with 35.8%, 55.6%, 7.3%, and 1.3% of all control rat kidneys

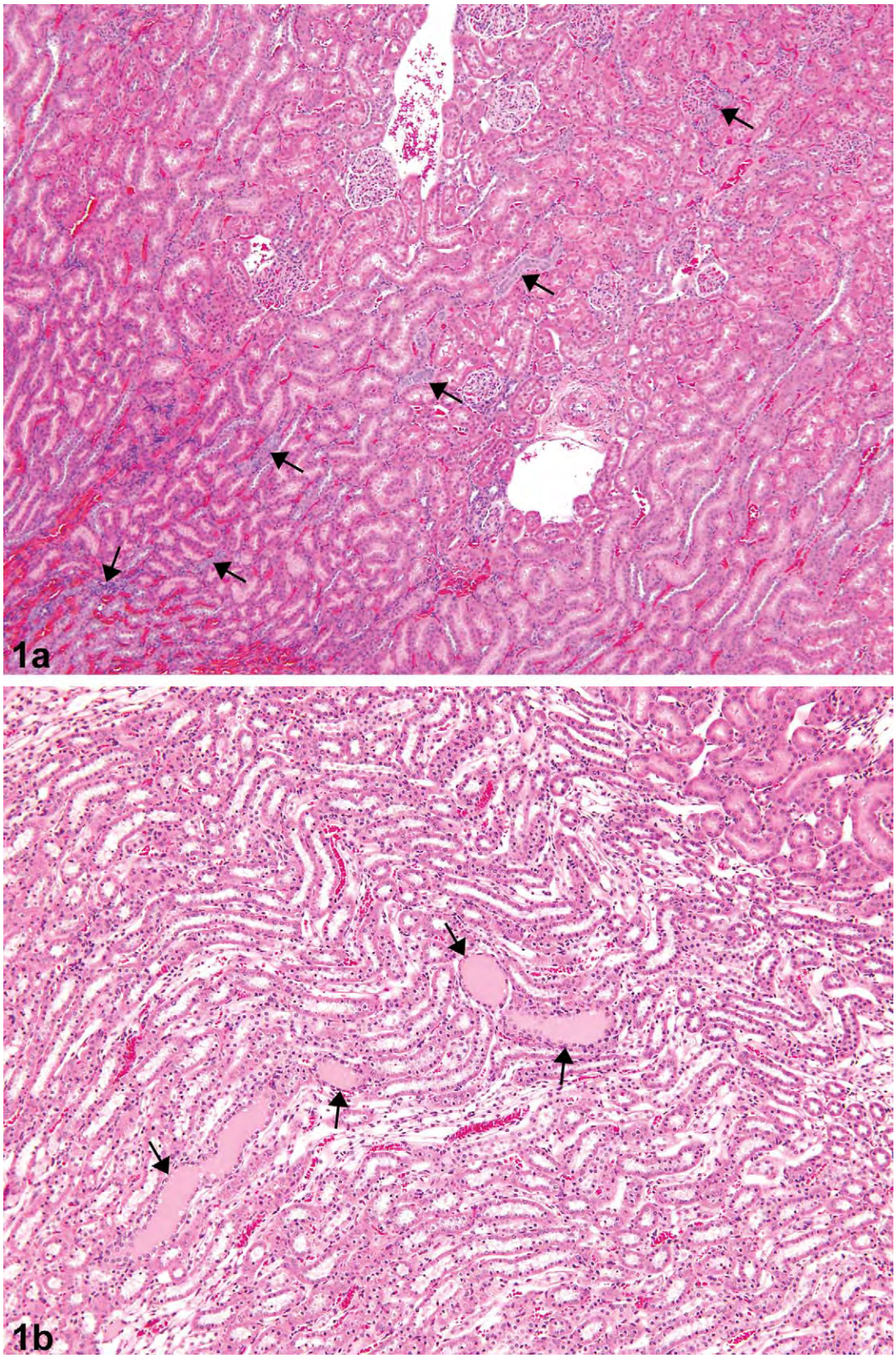

FIGURE 1.—(a) A series of basophilic tubule profiles representing a single nephron affected by chronic progressive nephropathy (CPN), extending in a linear fashion (arrows) from the cortex, through the outer stripe of the outer medulla, and down to the inner stripe of the outer medulla junction. Diagnosis of CPN could be based on any of the profiles indicated by an arrow. However, this particular lesion with its discontinuous profiles would be scored as only one focus of CPN. (b) A series of hyaline (or colloid) casts in the inner stripe of the outer medulla represents the turns of a single tubule. Hyaline casts in the inner stripe of the outer medulla or inner medulla are

TABLE 2.—Number of 90-day studies with chemical-related exacerbation, reduction, or no change in chronic progressive nephropathy (CPN).[a]

	Exacerbated	Reduced	No Change	Total Studies
NIH07 diet[b]	6	9	6	21
NTP2000 diet	9	5	7	21
Combined	15 (35.7%)	14 (33.3%)	13 (31.0%)	42

[a] Chemical-related change in CPN was determined by ± 0.5 differences in mean severity grades between the control and high-exposure groups.

[b] For the NIH-07 diet, one study (indium phosphide) was not evaluated for chemical-related change in CPN due to overriding non-CPN–related kidney lesions.

categorized as minimal, mild, low-moderate, and mid-moderate, respectively. However, the grade of CPN severity in control rats was significantly higher ($p < 0.0001$) in studies giving the NIH-07 diet (mean severity $\pm$ $SE = 2.0 \pm 0.04$) versus the NTP-2000 diet (1.5 ± 0.03). Of the control males fed NTP-2000, 54.4% had CPN categorized as grade 1 (minimal) compared with 16.4% for NIH-07, 0.4% of the males fed NTP-2000 had CPN categorized as grade 3 (low-moderate) compared with 14.6% for NIH-07, and only the NIH-07 diet had control male kidneys (2.7%) with a severity grade of 4 (mid-moderate). The inhalation route of exposure had the lowest grade of CPN among the control male rats, irrespective of diet. As CPN severity increased, absolute and relative kidney weights increased (data not shown).

Effects of Chemical Treatment on CPN Severity in 90-Day Studies

Regardless of diet (NIH-07 or NTP-2000), there were no differences in treatment-induced exacerbation or reduction of CPN in the high-exposure groups (Table 2). In addition, route of exposure was unrelated to exacerbation or reduction in CPN severity in the high-dose groups (data not shown). Fifteen chemicals, representing 35.7% of the total study set (6 NIH-07 and 9 NTP-2000), had an exacerbation of CPN severity in the high-exposure males, whereas 14 chemicals, representing 33.3% of the study set (9 NIH-07 and 5 NTP-2000), had a reduction of CPN severity, in some cases to grade 0. For 13 chemicals (31.0% of the study set; 6 NIH-07 and 7 NTP-2000), there was no change in CPN severity. For seven of the studies, the highest-exposure groups could not be evaluated for CPN, either due to low survival (benzophenone, urethane in water, dicyclohexylcarbodiimide, acrolein, and butanal oxime) or overriding non–CPN-related kidney lesions (3,3′,4,4′-tetrachloroazoxybenzene and indium phosphide). Consequently, for six of these seven chemicals, the next-to-highest dose group was examined and included in the statistical analysis. Indium phosphide was excluded because of a lack of an appropriate dose where CPN could be distinguished from chemically induced nephropathy.

CPN in 90-Day Studies and RTT Incidences at 2 Years

Twenty-four of the 43 chemicals evaluated in the 90-day studies proceeded to 2-year bioassays (Table 3), involving an equal number for each of the two diets. Of these 24 chemicals, nine (four NIH-07 and five NTP-2000) demonstrated a treatment-related exacerbation in CPN severity in the 90-day studies at the 2-year–equivalent dose (Table 3); seven of the nine chemicals had an exacerbation of CPN severity reported in the 2-year study. All seven chemicals (three NIH-07 and four NTP-2000) showing exacerbation of CPN in both the 90-day and 2-year studies demonstrated a significantly increased incidence of RTT (representing marginal-only increases in some cases) using the statistical methods described above. In addition, five of the seven chemicals with a significantly increased incidence of RTT had an increased incidence of renal hyperplasia; there was no instance in which increased renal hyperplasia occurred in the absence of increased RTT (Table 3). Four chemicals of the data set were considered by the NTP to be α_{2u}-g inducers, of which three were associated with a significantly increased incidence of RTT (Table 3). There were no apparent associations between increased RTT occurrence and chemicals considered genotoxic by the NTP (Table 3).

Since there was an association ($p < 0.0001$) between treatment-related exacerbation in CPN severity in the 90-day studies and increased incidence of RTT at 2 years, performance measures of the 90-day CPN findings as an indicator of increased RTT were calculated (Table 4). Based on this set of 24 studies, exacerbation of CPN at 90 days was a sensitive (100%) and specific (88%) indicator of increased RTT incidence at 2 years, predicting the presence or absence of increased RTT in a 2-year bioassay with 78% or 100% accuracy, respectively.

Other Treatment-Related Lesions

Apart from CPN effects, the most common treatment-related lesion in male rats of the forty-three 90-day studies was hyaline droplet accumulation in cortical proximal tubules (eight studies), usually accompanied by granular cast formation at the junction of outer and inner stripes of the outer medulla (six of the eight studies; Table 5). Of the twenty-four 90-day studies that proceeded to a 2-year bioassay, five had hyaline droplet accumulation (anthraquinone, citral, decalin, propylene glycol monobutyl ether, and Stoddard solvent IIC). The diagnosis of hyaline droplet accumulation/granular cast formation in the 90-day studies was positively associated with an increased incidence of RTT in the 2-year bioassays ($p = 0.015$). Although correspondingly specific (94%) for indicating increased RTT incidence at 2 years as CPN exacerbation (88%), hyaline droplet accumulation/granular cast formation at 90 days was not as sensitive (57%) as CPN exacerbation (100%).

Figure 1 (continued). characteristic of CPN and indicate more proximal (cortical) CPN involvement of the same nephron. The presence of an isolated cast of this type (arrows), without apparent association with a basophilic focus in the cortex, or linear basophilic tubules in the outer stripe of the outer medulla, would be counted as one CPN lesion.

TABLE 3.—Relationship between chemically-induced chronic progressive nephropathy (CPN) exacerbation and increased incidence of renal tubule tumors/hyperplasia, including NTP-reported genotoxicity and alpha$_{2u}$-globulin (α_{2u}-g) association.

CPN	Chemical Name	Tumors[a]	Hyperplasia[b]	Genotoxicity[c]	α_{2u}-g[d]
Exacerbated in 90-day and 2-year studies	Anthraquinone	Yes	No	+	
	Benzophenone	Yes	Yes		
	Decalin	Yes	Yes		+
	Divinylbenzene	Yes	Yes		
	Oxymetholone	Yes	No		
	Propylene glycol mono-t-butyl ether	Yes	Yes	+	+
	Stoddard solvent IIC	Yes	Yes		+
Exacerbated in 90-day study only	Citral	No	No		+
	p,p-dichlorodiphenyl sulfone	No	No	+	
Not exacerbated in either study	2-butoxyethanol	No	No		
	Chromium picolinate monohydrate	No	No		
	Coconut oil diethanolamine	No	No	+	
	Dibromoacetic acid	No	No	+	
	Diisopropylcarbodiimide	No	No	+	
	Elmiron	No	No		
	Formamide	No	No		
	2,4-hexadienal	No	No	+	
	Indium phosphide	No	No		
	Isobutene	No	No		
	Lauric acid diethanolamide	No	No		
	Methylene blue trihydrate	No	No	+	
	Oleic acid diethanolamide	No	No		
	Sodium dichromate dihydrate	No	No	+	
	Transcinnamaldehyde	No	No	+	

[a] Yes = a statistically significant trend and/or pairwise difference from the controls by the poly-3 test or by the hybrid test incorporating historical controls ($p \leq 0.05$); no = statistical analyses were not significant ($p > 0.05$).
[b] Yes = a statistically significant trend and/or pairwise difference from the controls by the poly-3 test ($p \leq \sim 0.05$); no = statistical analyses were not significant ($p > 0.05$).
[c] + = Chemicals considered by NTP to be genotoxic in one or more short-term assays.
[d] + = Chemicals considered by NTP to be candidates for acting via the α2u-g pathway.

DISCUSSION

CPN is regarded as a disease of old rats; however, the findings in this microscopic review of kidneys from 43 NTP 90-day studies provide confirmation that early stages of the disease can be observed in relatively young F344 males. Thus, lesions of CPN were detected at minimal to low-moderate levels of severity in 100% of the control male rats at approximately 4 to 5 months of age. Dixon, Heider, and Elwell (1995) reported similar findings for control male F344 rats from NTP 90-day studies (approximately 91% of the males reviewed); conversely, other reports have indicated no or low (<10%) incidence of CPN for male rats of approximately the same age as the animals reviewed here (Coleman et al. 1977; Maeda et al. 1985).

An explanation for this difference is most likely the specialized 0 to 8 grading scheme used to assess CPN in our reevaluation. Conventional schemes are based on estimating the percentage of parenchyma affected by CPN, using a qualitative 0 to 4 or occasionally a 0 to 5 grading scale (Coleman et al. 1977; Rao, Edmondson, and Elwell 1993). In one of these grading schemes, the lowest (minimal) grade represented involvement of less than 20% of the cortex and outer medulla by CPN, consisting of several small foci of renal tubular cell degeneration/regeneration and occasional tubules containing proteinaceous casts (Rao, Edmondson, and Elwell 1993). In the present study, however, a semiquantitative method was used based on a numerical count of focal lesions for the low grades (Hard and Khan 2004). For investigative purposes, incremental grading of CPN severity on a consistently measurable stage of disease progression is more suitable for elucidating subtle effects and detecting statistical differences than using a subjective assessment of percentage area of kidney involved. For example, a severity grade of 1 typically used in a conventional scheme may cover severity grades 1 to 4 in the expanded 0 to 8 grading system. Furthermore, for the chemicals evaluated in this review, the entity of CPN was not reported or scored by the NTP as a diagnosis in the 90-day studies, reflecting differences in grading methodology, which precluded any comparison between the NTP assessments at 90 days and the results reported here. Thus, the semiquantification of very low numbers of tubule lesions increased the sensitivity of the grading method, enabling identification of CPN-affected animals at the earliest histomorphologic stage. It should be emphasized that this 0 to 8 grading system is a specialized, labor-intensive research tool used for CPN-focused assessments only. It is not practical or recommended for use in routine histopathological evaluations of kidney sections in toxicity studies.

In this study, the type of diet had a significant effect on the severity of CPN in control male animals. While the incidence

TABLE 4.—Calculation of performance measures for exacerbation of chronic progressive nephropathy (CPN) in 90-day studies to indicate the increased incidence of renal tubule tumors (RTT) in 2-year studies.

	2-year bioassays		
	Number of studies with RTT[a]	Number of studies with no RTT	Total
Number of studies with exacerbated CPN[b] in the 90-day study	7	2	9
Number of studies with no CPN exacerbation in the 90-day study	0	15	15
Total	7	17	24
Performance measures			
Sensitivity (probability of exacerbated CPN with RTT)			7/7 = 100%
Specificity (probability of no exacerbated CPN with no RTT)			15/17 = 88%
+ Predictive value (probability of RTT with exacerbated CPN)			7/9 = 78%
– Predictive value (probability of no RTT with no exacerbated CPN)			15/15 = 100%
Efficiency (probability that the 90-day and 2-year results agree)			22/24 = 92%

[a] Increased incidence of RTT was determined by a statistically significant trend and/or pairwise difference from the controls by the poly-3 test or by the hybrid test incorporating historical controls ($p \leq 0.05$).

[b] Chemical-related exacerbation in CPN was determined by a $\geq$0.5 difference in the mean severity grades between the control group and the dose group in the 90-day study that was identical to, or most closely approximated, the top dose used in the 2-year bioassay.

TABLE 5.—Chemical-related kidney lesions other than chronic progressive nephropathy (non-CPN) observed in the histopathological reevaluation of forty-three 90-day NTP studies.[a]

	All 90-day studies[b]	90-day studies proceeding to 2-year bioassays[c]
Number of studies evaluated	43	24
Number of studies with lesions	19	7
Lesion		
Hyaline droplet accumulation[d]	8	5
Tubular pigment	7	1
Granular casts or precursors	6	5
Mineralization[e]	3	1
Glomerulopathy	2	
Nephrosis	2	
Cytoplasmic vacuolization	1	
Linear papillary mineralization	1	
Papillary necrosis	1	
Pyelonephritis	1	
Tubular hyperplasia	1	

[a] Data are reported as number of studies with the lesion.

[b] Kidney sections from control and high-exposure male rats were evaluated for evidence of treatment-related non-CPN lesions.

[c] Kidney sections from control males and treated male rats from the 90-day study whose dose approximated the highest dose used in the 2-year bioassay were evaluated for evidence of treatment-related non-CPN lesions.

[d] Hyaline droplets were consistent with α_{2u}-globulin in five of eight studies and four of five studies in the "all" and "2-year" study sets, respectively.

[e] Mineralization was intratubular and located at the outer stripe of the medulla/inner stripe of the medulla junction.

of CPN was not altered, the severity was 25% lower in control male animals on the lower protein diet (NTP-2000) compared with the higher protein diet (NIH-07). This is consistent with the beneficial kidney effects previously reported for the NTP-2000 diet. Rao, Edmondson, and Elwell (1993) demonstrated that decreasing the dietary protein concentration from 23% to 15% reduced the severity of CPN in male rats at 2 years by approximately 50%. Our study shows that the reduction in CPN severity associated with feeding a low-protein diet commences early (at least by 4 to 5 months of age). Furthermore, there was a positive correlation between CPN severity and absolute and relative kidney weights regardless of diet. Such a correlation suggests that the presence of CPN lesions augments the weight of the organ, even at an early stage of lesion development. This would be in keeping with the fact that end-stage CPN kidneys are increased in size, in contrast with end-stage kidneys from various causes in humans, where typically there is shrinkage of the organ (Hard, Johnson, and Cohen 2009).

No previous reports have estimated the frequency with which chemically induced CPN exacerbation in safety assessment studies occurs. Treatment-related exacerbation of CPN was observed in approximately one-third of the 43 studies evaluated here. Conversely, treatment-related reduction of CPN occurred at a similar rate. Thus, treatment-related alterations in CPN, either exacerbation or reduction, can be a common finding (70%) in 90-day studies. Whereas exacerbation of CPN is regarded as an adverse event, reduction of CPN may likely be a response to treatment-related stress. The structures of chemical agents known to exacerbate CPN are diverse, and a mechanism for the enhancing effect is not known (Doi et al. 2007). Since the expression of CPN can be modified by nutritional factors (reviewed in Hard and Khan 2004), a difference in rates of treatment-related CPN exacerbation/reduction between the NTP-2000 and NIH-07 diets might have been expected in this study, but there was no evidence for such a dietary role. Thus, chemicals destined to exacerbate CPN apparently did so whether a low- or high-protein diet was used.

Our investigation showed a strong association between CPN exacerbation at 90 days and subsequent RTT occurrence. After statistical recalculation, all of the seven chemicals demonstrating an increased incidence of RTT in the 2-year bioassay (see Table 3) had a treatment-related exacerbation in CPN at both 90 days and 2 years. In addition, five of these same seven chemicals had an increased incidence of tubule hyperplasia, a lesion considered by NTP (NTP 2006) and others (Hard 1987; Lipsky and Trump 1988; Dietrich and Swenberg 1991; Nogueira, Cardesa, and Mohr 1993) to be on a developmental continuum with RTT. There was no increase in hyperplasia for any of the remaining 24 chemicals taken to a 2-year endpoint. Consequently, the data on tubule hyperplasia supported the association between CPN and increased incidence of RTT (see Table 3).

The notion that increases in CPN severity in chronic studies can be associated with increased frequency of RTT is not new. Anver et al. (1982) reported pathology data from two stocks of untreated Sprague-Dawley rats held into senescence, indicating that the stock with the higher incidence of spontaneous renal adenoma also had the highest severity of CPN over comparable age groups and life span. In other work, it has been noted that an increase in RTT frequently occurred in dose groups exhibiting chemical exacerbation of CPN (Montgomery and Seely 1990). In their characterization of the utility of multiple-section sampling and evaluation of the kidney for carcinogenicity studies, Eustis et al. (1994) used, as one of their criteria for study selection, a chemical-related increase in the average severity of CPN. This criterion was included because, in their experience, slight or marginal increases in renal tubule hyperplasia and/or adenoma often accompanied chemical-related exacerbation of CPN. Furthermore, they noted that a chemical-related increased severity of nephropathy was seen in male rats for every study in which step-section evaluations were useful in establishing a relationship between chemical administration and increased incidences of renal proliferative lesions. More recently, Seely et al. (2002) demonstrated a slight, but statistically significant, association between increased severity grades of CPN in male F344 rats with RTT compared with age-matched animals without tumors. A statistically significant positive association of RTT with an advanced stage of CPN has now been shown for carcinogenicity studies of hydroquinone (Hard et al. 1997), ethyl benzene (Hard 2002), and quercetin (Hard et al. 2007). The findings reported here add further support to the work cited above.

A novel aspect of our data is that chemically induced exacerbation of CPN at 90 days may be a predictive indicator of RTT occurrence at 2 years. In a recent investigation sponsored by International Life Sciences Institute seeking biological endpoints predictive of a tumorigenic outcome (Boobis et al. 2009), a kidney weight increase at 13 weeks was the only finding that was consistently associated with renal tumors. Although our results suggest that chemically induced exacerbation of CPN at 90 days also has that potential to predict a subsequent increase in RTT (see Table 4), there are some caveats to be considered. First, the selection criteria used for this evaluation resulted in the identification of studies that, in general, had been performed on industrial chemicals or environmental/food contaminants; no studies involving drugs in development, small molecules, dietary supplements, or physical agents were included. Second, the seven chemicals associated with RTT may not have represented a broad enough range of modes of action operative in renal carcinogenesis (Hard 1998; Lock and Hard 2004). Although anthraquinone is regarded as a genotoxic chemical (NTP 2005), it was of one of five chemicals that induced hyaline droplet accumulation at 90 days. Hyaline droplet nephropathy is known to be accompanied by CPN exacerbation (Hard et al. 1993), and this association may have skewed the results in favor of demonstrating a correlation between subchronic CPN exacerbation and chronic RTT occurrence. In addition, as CPN is characterized by a highly elevated proliferative rate (Short, Burnett, and Swenberg 1989), a synergistic interaction between hyaline droplet nephropathy and advanced CPN resulting in a marginal increase in RTT incidence is plausible (Lock and Hard 2010). Finally, female rats were not included in the study. Therefore, at this stage, our findings should be regarded as pertinent to the male rat only. As rat CPN has no counterpart in humans (Hard et al. 2009), RTT arising in association with chemically induced CPN exacerbation should have no relevance for species extrapolation in human risk assessment.

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ATTACHMENT F:

Weiler, E., Farbman, A.I. (1997) Proliferation in the rat olfactory epithelium: age dependent changes. J. Neurosci. 17, 3610-3622.

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Proliferation in the Rat Olfactory Epithelium: Age-Dependent Changes

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Vertebrate olfactory sensory neurons are replaced continuously throughout life. We studied the effect of age on proliferation in olfactory epithelium in postnatal rats ranging in age from birth (P1) until P333. Using BrdU to label dividing cells, we determined the proliferation density of basal cells, i.e., the number of labeled nuclei/unit length (240 μm) of olfactory epithelium in coronal sections from six different anterior–posterior levels from each animal. A total length of >1 m of olfactory epithelium was counted in each age group. We observed a dramatic decrease of proliferation density from P1 through P333. At P1, proliferation density is 151 cells/mm; it decreases to approximately half at P21 (70 cells/mm), and half again at P40 (37 cells/mm). At P333 the proliferation density was only 8/mm, ~5% of that seen at P1. The changes were clearly related to age and not to body weight, because the values were essentially identical for males and females of the same age but of different body weight. Proliferating cells appear in patches that, after P40, become more separated from one another and contain fewer cells. In 6- and 11-month-old rats, 30 and 45% of all units contained no labeled cells. We confirmed the data of others that the olfactory surface area continuously increases with age; we showed that there is a reciprocal relationship between proliferation density and surface area. The proliferating cells provide neurons to sustain growth as well as to replace dying cells.

Key words: olfactory epithelium; proliferation density; basal cell; development; BrdU method; growth and replacement; turnover; mitosis; neurogenesis; age

Olfactory sensory neurons are replaced continuously throughout life in vertebrates. The uniqueness of the olfactory system lies in the fact that a progenitor population of globose basal cells (cf. Mackay-Sim and Kittel, 1991b; Schwartz Levey et al., 1991; Suzuki and Takeda, 1991) can divide and differentiate and, therefore, can reconstitute the sensory epithelium, even under physiological conditions (Moulton et al., 1970; Thornhill, 1970; Graziadei and Metcalf, 1971; for review, see Farbman, 1992). The average life span of olfactory neurons was thought to be ~1 month (Moulton, 1975; Graziadei and Monti Graziadei, 1979), although some evidence suggests they live considerably longer (Hinds et al., 1984; Mackay-Sim and Kittel, 1991a,b). In rodents, the life span varies from ≤24 hr (Carr and Farbman, 1992) to >1 year (Hinds et al., 1984).

Neurogenesis has been studied primarily in the "wound-healing" model (after massive cell death is experimentally induced). After ablation of the olfactory bulb or axotomy of the olfactory nerve, there is an increase in proliferation density, i.e., the number of cells labeled with a DNA precursor/unit length or volume of epithelium (Schwartz Levey et al., 1991; Suzuki and Takeda, 1991; Carr and Farbman, 1992). Upregulation of the mitotic rate can also be induced by destroying the epithelium with zinc sulfate (Cancalon, 1982), methyl bromide (Schwob et al., 1995), or *N*-methyl-formimino-methylester (Rehn et al., 1981). In recovery, the mitotic rate is increased over control levels. On the other hand, the occlusion of one nostril in a newborn rat results in a downregulation of the proliferative rate on the side ipsilateral to the occlusion (Farbman et al., 1988; Cummings and Brunjes, 1994).

In all of these reports, the possible influence of age on proliferation density was virtually ignored. Smart (1971) was the first to note, in passing, that there "... appeared to be a progressive decrease in the frequency of mitosis with age ..." (up to postnatal day 58). In a direct examination of the influence of age on proliferation density in mice, Dodson and Bannister (1980) reported a lower index (tritiated thymidine-labeled cells/total cells) with age, but only three age groups were examined, 19 d, 6 months, and 1.5 years. In other studies, the reported data revealed mitotic rate decreases in older control animals, but the authors either took no special note of it (Cummings and Brunjes, 1994; Holtmaat et al., 1995) or simply mentioned it in passing (Hinds et al., 1984; Paternostro and Meisami, 1994).

In the present study, we report that proliferation density in the olfactory epithelium of unperturbed rats declines dramatically with age (irrespective of body weight) from the neonate to the age of 11 months. Further, we argue that the data are consistent with the hypothesis that the life span of individual olfactory neurons increases with age.

MATERIALS AND METHODS

Animals. Sprague Dawley rats of both sexes were used at postnatal ages ranging from P1 (day of birth) to P333 (Table 1). They were bred in our colony and housed in a temperature-controlled environment with a 12 hr light/dark cycle and access to food and water *ad libitum*. Newborns were allowed to suckle until day 40 (weaning). During that time, they were weighed every other day. After weaning, animals were separated by sex and kept in groups. From then on, they were weighed twice a week. Animals from different litters were used in each age group.

BrdU injection, perfusion, and fixation. To label dividing cells, animals were given a single dose (50 mg/kg body weight) of BrdU (5-bromo-2′-deoxyuridine, Sigma B 5002, St. Louis, MO) intraperitoneally in a solu-

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Table 1. Proliferation density in the olfactory epithelium during postnatal development measured as BrdU-labeled basal cells per length

Age	Number of animals m	f	N	Cells per mm	SE	Significance p value*	Counted units	Counted mm
P1–3	2	2	4	150.88	4.51		662	158.09
P11	0	1	1	118.65			415	99.10
						0.002**		
P21	4	2	6	69.85	2.19		5075	1211.91
						0.0001		
P40	4	5	9	36.81	3.04		5340	1275.19
						0.0001		
P66	3	4	7	24.24	1.44		4264	1018.24
						0.001		
P105	4	1	5	16.80	0.25		4271	1019.91
						0.018		
P181	3	0	3	10.16	0.76		3912	934.19
						0.05		
P333	3	0	3	8.03	0.24		5093	1216.21

m = males; f = females; N = total number of animals.

*Mann-Whitney *U* test, U = 0 among all age groups; the values did not overlap between age groups, so that *p* depends only on the number of animals.

**P1–3 and P11 were combined as one group for the calculation of the *p* value.

tion of 20 mg/ml PBS, pH 7.2. The injection was given between 8:00 A.M. and 10:00 A.M. One hour later, rats were killed by intraperitoneal injection of a lethal dose of sodium pentobarbital (250 mg/kg body weight). While under deep anesthesia, the animals were perfused (with a perfusion pump) intracardially through the left ventricle with 0.1 M PBS, pH 7.2, at room temperature (RT) to clear the vessels of blood. The total flow of perfusate was 1 ml/gm body weight; the rate of flow was adjusted to take 5 min. This was followed by perfusion of a fixative solution, 4% paraformaldehyde in 0.1 M Sørensen's phosphate buffer, pH 7.0 (1 ml/gm body weight, 20 min, RT). Heads were removed, skinned, and post-fixed overnight in the same fixative at 4°C. After washing several times in 0.1 M Sørensen's phosphate buffer, pH 7.0, they were processed for histology.

Histology (decalcification, embedding, sectioning). As much of the bone as possible was removed before the noses were decalcified in 5% EDTA in 0.1 M Sørensen's phosphate buffer for several days, with daily changes of solution. After decalcification, the specimens were washed in distilled water, dehydrated in increasing concentrations of ethanol and transferred to Histosol (National Diagnostics, Atlanta, GA) to clear the tissue. The specimens were infiltrated with paraplast and embedded. Frontal (coronal) sections of 10 μm were cut serially from the tip of the nose to the most posterior extension of the olfactory epithelium, and each section was preserved.

Staining. Every 10th section was placed on silane (3′-aminopropyltriethoxy-silane, Sigma A-3648) treated slides. Sections were deparaffinized, rehydrated to water, stained with hematoxylin and eosin (H&E), dehydrated again, mounted permanently with Permount, and coverslipped. These sections were used to demonstrate the extent of the olfactory epithelium and to determine the regions (see below), as well as to measure the area and thickness of the epithelium and the cell density.

BrdU immunohistochemistry. After 2 hr of UV light activation (our unpublished results), sections were rehydrated and treated with trypsin (0.1% trypsin, Sigma T-8642, 0.1% $CaCl_2$ in 0.05 M Tris buffer for 10 min at room temperature) to increase the signal of the antibody reaction (Hayashi et al., 1988) and washed afterward several times in PBS. Sections were then incubated sequentially at 37°C in a 1:1 mixture of normal horse serum and 0.1 M PBS to block nonspecific binding (60 min), an antibody against BrdU [undiluted Amersham anti-bromlodeoxyuridine solution, containing mouse monoclonal antibody and a nuclease, Amersham RPN 202, Amersham Life Science, Arlington Heights, IL (60 min)], the Vectastain Elite Kit for mouse antibodies (Vector Labs, Burlingame, CA) containing biotinylated horse anti-mouse secondary antibody (30 min) and the ABC reagent (45 min). Each antibody treatment was followed by a 15 min wash with PBS at room temperature. Specimens were then incubated for 5 min at room temperature in a freshly made solution containing 0.01% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine in 0.1 M Tris-HCl buffer, pH 7.5, to reveal the BrdU immunoreactivity. To stop the reaction, specimens were placed in distilled water, washed several times, and dehydrated in increasing concentrations of ethanol before being mounted permanently with Permount and coverslipped.

Selection of sections. The sections used to determine the area of the olfactory sheet were taken from the H&E stained series at equidistant intervals, appropriate for the age, from the entire anterior–posterior extent of the olfactory epithelium.

For measuring the thickness of the olfactory epithelium, the H&E-stained sections were used. To avoid artifactual thickness measurements at the edges, where the turbinates either are first visible in a section or are seen to fuse, only sections where the turbinate was fully developed were used for measurements. These sections were also used to determine the cell density.

The immunohistochemical procedure for determining the proliferation density was applied to sections from six different regions, ranging from anterior to posterior. Because the turbinates develop allometrically and not symmetrically, we used sections from the following regions defined by the pattern of the turbinates rather than sections at equidistant intervals (Fig. 1):

Region 1: Where the first appearance of the turbinates is located in the section (the dorsal part of the second endoturbinate, appears first).

Region 2: The first and second ectoturbinates as well as the second endoturbinate, are well represented and the third ectoturbinate just starts to appear.

Region 3: All turbinates are fully visible.

Region 4: The second endoturbinate fuses with the bone on the dorsal part of the nasal cavity.

Region 5: The third endoturbinate fuses with the bone of the dorsal part of the nasal cavity.

Region 6: The fourth endoturbinate fuses with the bone of the dorsal part of the nasal cavity.

These regions could be observed and defined in rats aged ≥40 d. In 11- and 21-d-old animals, region 3 was defined as the full appearance of all turbinates except the fourth, but before any turbinate fusion. In younger animals the regions could not be defined in this manner, because the turbinates are not as fully developed. In these cases, the first and last regions were determined and sections from intervening regions were taken at equidistant intervals, to give a total of six regions.

Measurements. For all measurements and counts except cell density, we used an ocular micrometer and a magnification of 400× or 1000×. At these magnifications, each of the 100 units on the ocular micrometer scale represented 2.4 or 1.0 μm, respectively. Thus, in the BrdU study, we counted the number of BrdU-positive cells in a 240 μm unit of epithelial length.

Area of the olfactory epithelium. To confirm that the areas of olfactory epithelium in our rats increased with age (Hinds and McNelly, 1981;

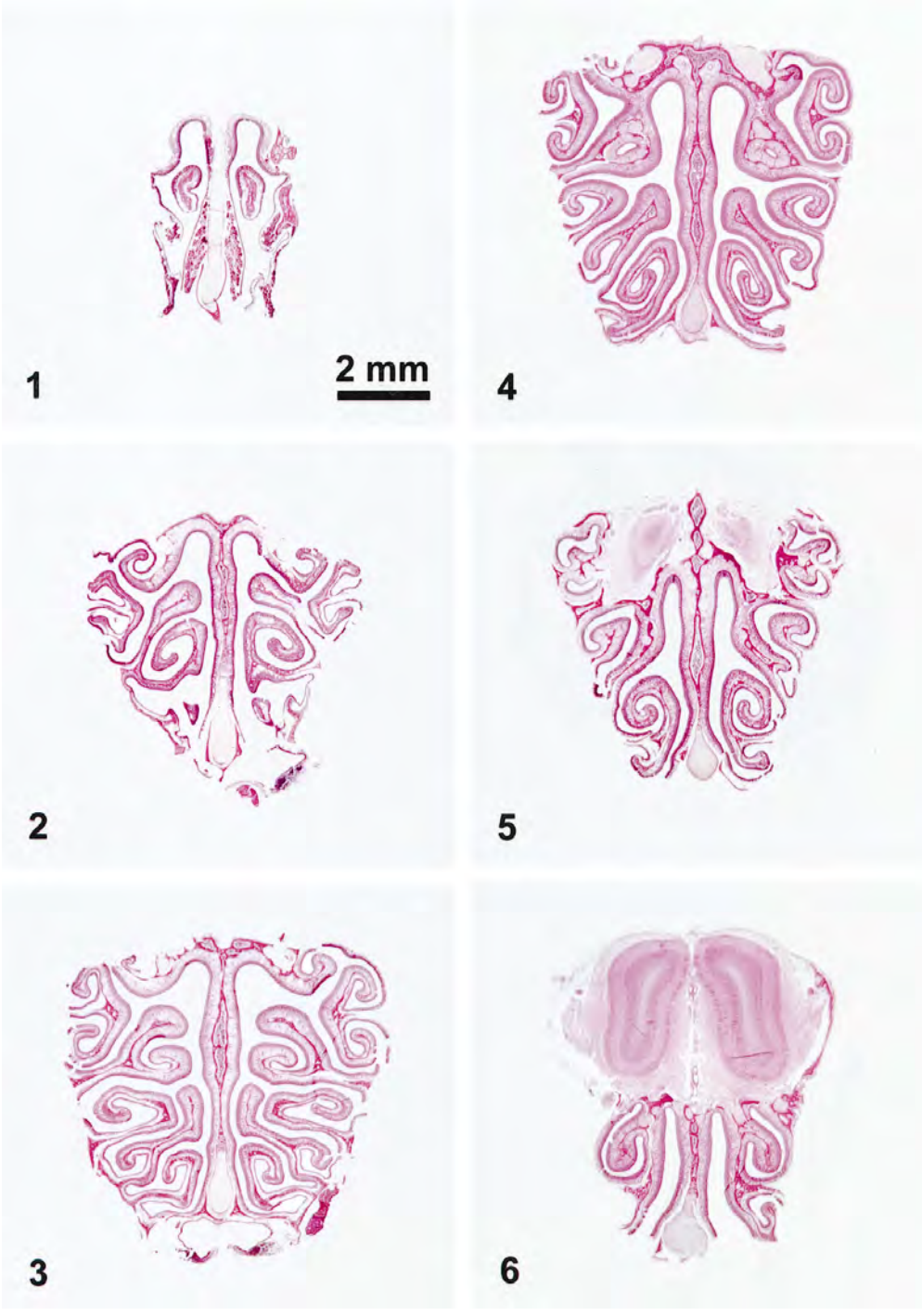

Figure 1. Coronal sections from the rat nose at different anterior–posterior levels. These six regions, defined by the pattern of the turbinates were used in the present study.

Meisami, 1989; Apfelbach et al., 1991; Paternostro and Meisami, 1993), we measured the area of the olfactory sheet on the right side in sections from one randomly selected male rat in each age group. By tracing along the olfactory epithelium of the H&E-stained frontal sections, the epithelial length was measured at equidistant intervals along the anterior–posterior axis (for P1, every 200 μm; for P21 and P40; every 400 μm; for all others, every 500 μm). The measurements were graphed and the areas under the curves were calculated.

Epithelium thickness. The thickness of the olfactory epithelium was measured on the right side of H&E sections adjacent to those on which the immunostaining was performed. The measurements were taken at 240 μm intervals on each section all around the whole septum and turbinates. With this procedure, ~100 values per section were collected. The measured thickness was from the basement membrane to the top of the knobs. The values of all measurements of the sections for each individual animal were averaged, and the mean value for the animals in each age group was calculated.

Cell density. To determine cell density, the number of nuclei in a 200 μm length of epithelium was counted with a computerized morphometric system, ANALY*SIS,* connected to a CCD camera on a Zeiss (Oberkochen, Germany) photomicroscope. (We are grateful to Dr. R. Apfelbach, University of Tübingen for permission to use this system.) The thickness of the epithelium of each sample was measured at the middle of the length, and the supporting cells, basal cells, and neurons were counted as separate populations. We used the position, staining pattern, and shape of the cell nucleus to discriminate the cell types. We realize that it is not always possible to discriminate between a very young neuron and a globose basal cell, but attempted to be consistent in applying our criteria to all specimens; thus, the counts of basal cells and neurons should be regarded as reasonable estimates. The number of basal cells

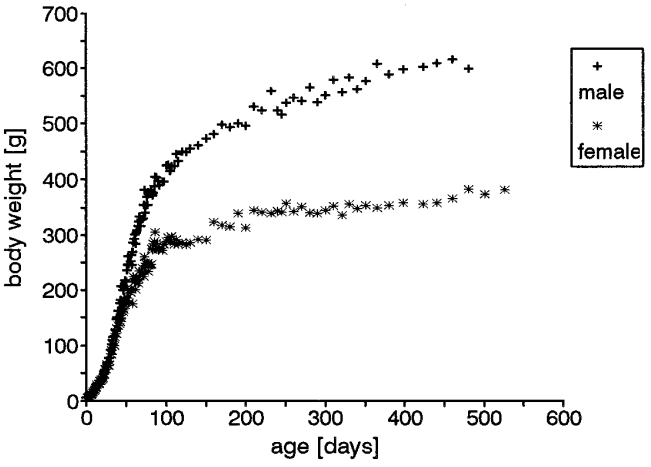

Figure 2. Postnatal changes in the mean value of the body weight from male and female Sprague Dawley rats. Body weight increase continues with age (during the period studied) in males as well as in females. The females show a significantly slower increase after P25 compared with males.

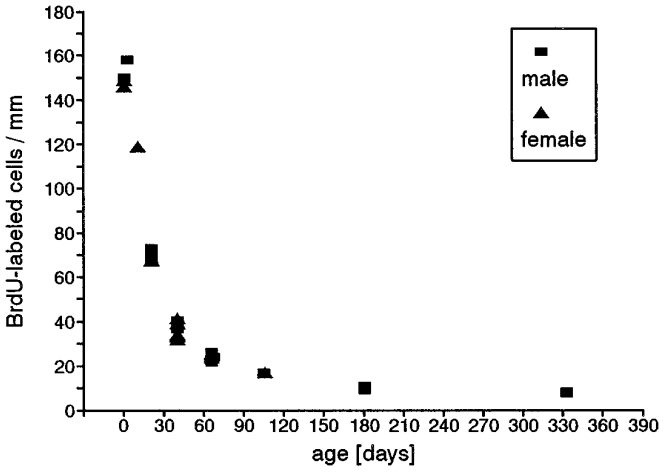

Figure 3. Proliferation density in the olfactory epithelium measured as the number of BrdU-labeled basal cells per millimeter. Each *symbol* represents the average value of one animal. In some age groups, the values are so close that they cannot be distinguished as separate symbols. For detail (animal numbers), see Table 1. The values for males and females at the same age are not different from one another.

was estimated by counting the number of nuclei in the basalmost layer of epithelium, whether they were round or flat. The number of neurons was estimated by counting all suprabasal round nuclei in the epithelium, and the number of oval-shaped supporting cell nuclei in the most superficial nuclear layer was estimated. Repeat counts of selected specimens demonstrated that our method was reproducible. Approximately 16–17 samples were taken from the septum and turbinates in each animal. Samples were chosen randomly at different epithelial thicknesses. We had ascertained earlier that the nuclei of the neurons in the different age groups were the same size. We did not use a correction factor (Abercrombie, 1946; Coggeshall and Lekan, 1996), because there was little variation in nuclear size or section thickness. Another reason for not using correction factors was that in the counts of BrdU-positive cells, the stained nuclei appeared larger because the marker often spread beyond the nuclear margins and it was not possible to discriminate easily between a nucleus cut through its center or closer to its margin. Because we wanted to correlate the BrdU counts and the cell density counts, we could not correct one without the other.

Proliferation density. To determine the proliferation density, the numbers of BrdU-labeled basal cells were counted along 240 μm length units of the olfactory epithelium over the whole section in each region. Except for the newborns, between 400 and 1200 units (between 100 and 300 mm of olfactory epithelium) for each animal were counted. Right and left sides were counted separately in each region, and the septum and turbinates of each region were counted separately. For each age group except the neonates, a total length of ~1 m of olfactory epithelium was evaluated (Table 1).

Analysis of data. To analyze the proliferation density (number of labeled basal cells per unit length epithelium), several parameters were calculated.

For each animal the proliferation density of the different regions (anterior–posterior extent) as well as of the septum and the individual turbinates was averaged separately. Because there were no differences between values for septum and turbinates, these values were combined and averaged within each age group.

To obtain a mean value for the whole olfactory epithelium of an animal, all counted values were taken and averaged. These mean animal values were used to calculate the mean value of the age group.

In addition, the specimens were analyzed for the distribution pattern of the proliferating cells as follows. All values of the proliferation density per unit were ordered and the percent frequency for each density calculated for each animal. The distribution pattern was graphed for each animal. The mean values of the percent frequency were calculated for each density to give the distribution pattern of the age group.

For each animal, right and left side were measured and counted separately and compared.

The data for males and females were compared for each age group.

Statistics. We used the nonparametric Mann–Whitney *U* test to determine whether there were differences in the cell and proliferation densities among age groups, as well as differences between the right versus left side, females versus males, and among the antero-posterior regions (Lienert, 1973). The epithelial thickness measurements were performed only on the right side, and the Mann–Whitney *U* test was used to determine whether there were differences in thickness among groups. To analyze differences in the distribution pattern, we used the Kolmogoroff–Smirnoff test (Sachs, 1982).

RESULTS

Age-dependent change in proliferation

The body weight of rats continues to increase for most of their lives, until senescence (Fig. 2), and the total surface area covered by olfactory epithelium also increases (see below, Fig. 8; Hinds and McNelly 1981). In this part of the study, we examined the rate of proliferation as a function of age by injecting rats of different ages with BrdU and killing 1 hr later.

Proliferation density in the olfactory epithelium, measured as the number of BrdU-labeled basal cells per millimeter length, showed a dramatic asymptotic decrease as the animals became older (Fig. 3, Table 1). In neonates, proliferation density was high, with an average value of 151 labeled cells/mm. At P21, the value decreased to approximately half this value (70 cells/mm), and to half again at P40 (37 cells/mm). It is important to note that the density still decreased in the adult (P66, 24 cells/mm), reaching values of only ~10% of that of newborns at P105 (17 cells/mm). The proliferation density did not reach a plateau, but decreased again between 6 months (10 cells/mm) and 11 months, an additional reduction of 20% was observed, reaching only 8 cells/mm, half the value of that at P105. The differences in the proliferation densities among all age groups were highly significant (Table 1).

Distribution pattern: dividing cells appear in clusters

We ordered the data in terms of frequency of occurrence by plotting the number of labeled basal cells counted within each unit length of epithelium in a single microscopic field (each unit was 240 μm long at 400× magnification). Figure 4 is a graph showing the distribution patterns for each age group. Along with the age-related decrease in proliferation density, there was a reduc-

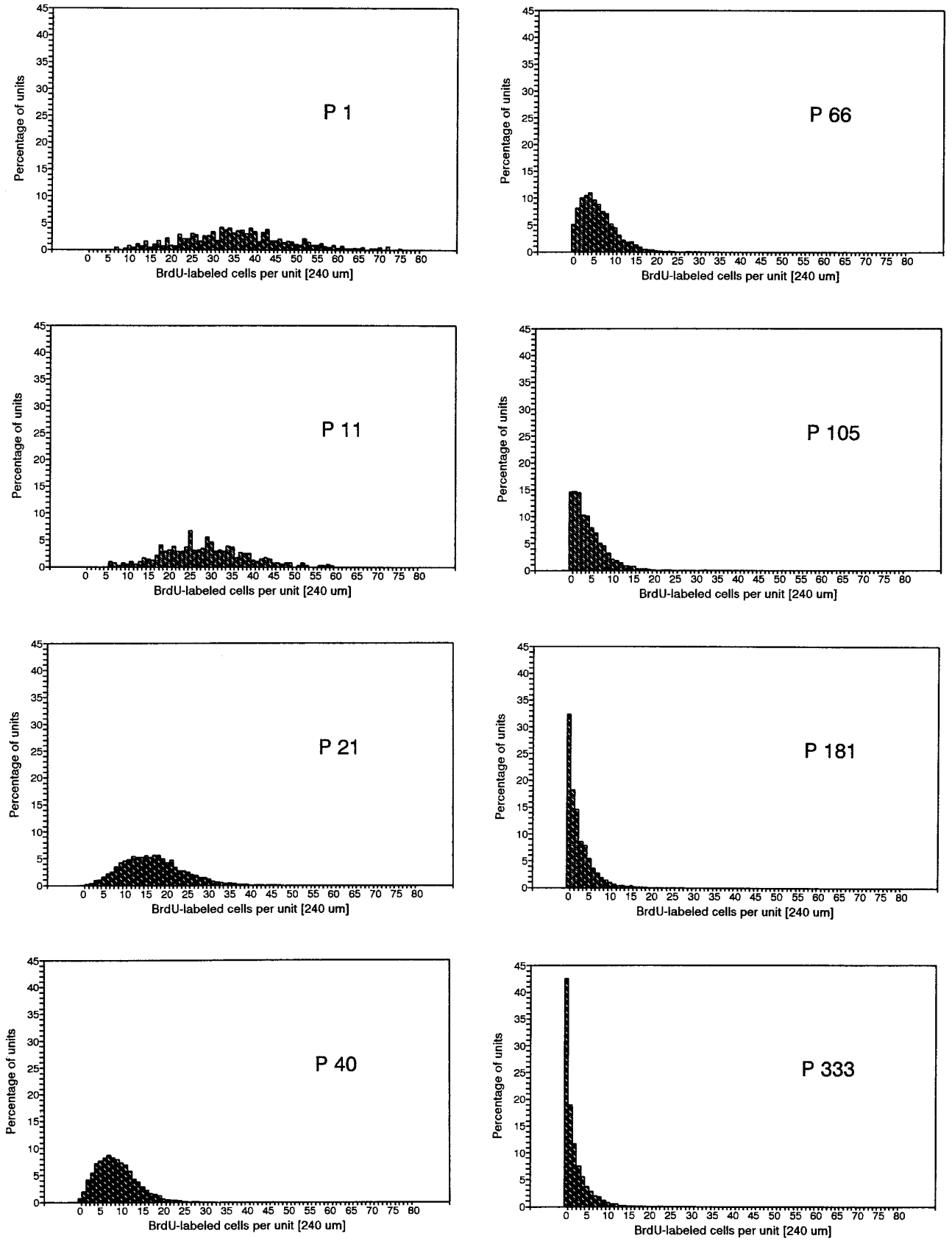

Figure 4. Distribution pattern of the proliferation density. In young animals, more units (240 μm) have a high number of labeled cells, whereas in older animals most units have only a few or no labeled cells.

Table 2. Proliferation density in the different regions ranging from anterior to posterior

Age	Region 1	Region 2	Region 3	Region 4	Region 5	Region 6
P1–3	152.74 ± 17.96	157.63 ± 18.19	156.49 ± 7.67	121.02 ± 19.35	153.40 ± 9.51	151.63 ± 2.88
P11	114.25	118.99	120.83	115.00	122.36	116.07
P21	73.77 ± 7.97	71.19 ± 6.05	69.32 ± 2.35	68.71 ± 3.76	69.86 ± 2.13	70.55 ± 2.65
P40	41.21 ± 8.41	37.28 ± 3.19	36.35 ± 3.87	36.44 ± 3.44	36.02 ± 3.17	37.52 ± 3.66
P66	31.79 ± 7.69	24.10 ± 2.07	23.21 ± 2.32	24.39 ± 1.57	24.31 ± 2.59	24.75 ± 2.32
P105	20.00 ± 5.42	16.95 ± 2.34	15.91 ± 0.81	17.03 ± 1.46	16.81 ± 1.35	17.70 ± 2.61
P181	15.82 ± 2.60	11.66 ± 1.37	9.64 ± 0.15	9.07 ± 1.42	10.10 ± 1.21	10.58 ± 1.89
P333	14.71 ± 2.19	9.26 ± 0.45	8.35 ± 0.28	7.07 ± 0.43	7.10 ± 0.28	7.05 ± 0.64

Mean values and SE for each age group.

tion in the relative number of labeled basal cells counted in each unit. In sections from neonates, we observed a high frequency of units containing >30 labeled cells; in contrast, such high values were never seen in old animals. On the other hand, newborn animals showed no units with zero labeled cells, whereas in animals older than P180, >30% of all units did not contain a single labeled cell. The shape of the distribution pattern changed in an age-dependent manner and the patterns were highly significantly different from one another (Kolmogoroff–Smirnoff test, $p < 0.001$).

One of the characteristics of the distribution of proliferating cells at P40 and later was their distribution in clusters or patches (cf. Moulton et al., 1970; Monti Graziadei and Graziadei, 1979). In newborns (Fig. 5*A,D*), the labeled cells were so densely packed that they sometimes spanned nearly two rows, and there was hardly any space between them. With increasing age, the labeled cells lined up in a nearly continuous band (Fig. 5*B*) and later appeared in clusters (Fig. 5*C,E,F*), first observable at P40. The clustering of labeled cells became progressively easier to observe at P66 and older. However, in the oldest animals (P333), the number of cells within a cluster became smaller and sometimes extents of epithelium several millimeters long were found without a single labeled cell (Fig. 5*C*). This is reflected in Figure 4 by the high frequency of units (>40%) with zero labeled cells and the high frequency of units (>40%) with few (1–5) labeled cells.

Regional and area differences

We have noted that the proliferating cells appeared in patches and, further, the number of labeled cells per unit from one 240 μm unit to another varied widely in the same animal and in the same section. Moreover, among the six anteroposterior regions taken from an *individual animal*, the local differences in the proliferation density varied by as much as 30%. Nevertheless, the *average* values among five of the six regions ranging from anterior to posterior showed no differences in proliferation density in all age groups (Fig. 6, Table 2). The only exception was the most anterior region; beginning at P21 and in all older age groups, the count in the anterior region was slightly higher. In the age groups P181 and P333, the proliferation densities in the most anterior region were significantly higher than those in the other five regions ($p < 0.005$). Moreover, region 1 in each age group showed the most variability. This is consistent with the fact that this region is most vulnerable to damage, i.e., most exposed to drying out and to the effect of airborne pollutants or potential cytotoxic agents and infectious organisms from the environment. If more cells in this region die, there would be a greater demand for replacement cells. Moreover, much of the incremental growth in area would also occur in that region. That growth occurs anteriorly is reflected in the area profiles (Fig. 8); the anterior part of the profile becomes longer in older rats, and the major region of olfactory epithelium shifts posteriorly.

We analyzed the proliferation density in the different areas within a region, e.g., the septum versus the different turbinates. The number of BrdU-labeled basal cells in the septum generally was lower than the average of the entire section in a given region. Lower values were also seen in the second and third endoturbinates, compared with the ectoturbinates. This occurred more frequently in younger age groups than in older ones. These differences, however, were not statistically significant.

Sex differences

Despite the fact that the males and females showed a different rate of growth from P25 onward, and even though the differences in their body weights after that age were statistically significant (Fig. 2), no differences in the proliferation density (Fig. 3) or distribution pattern at any investigated age were detected.

Differences between right and left sides

Right–left side differences in the density of BrdU-labeled basal cells for individual animals ranged from −16% to +14% (Fig. 7), if the value for the right side is arbitrarily set at 100%. The variations in values between right and left sides of regions or even single sections were sometimes even greater; however, there was no pattern suggesting that one side was consistently higher than the other in any age group. The percent differences were larger in older animals, probably because the values were lower and a small absolute difference made a high relative difference. These variations might be related to the nasal cycle, i.e., the cyclic (~2–3 hr), autonomically controlled partial closure of one naris. Although the differences between right and left seemed high in individual animals, the differences in the proliferation density *within any age group* were not statistically significant.

Proliferation density of other structures

Although we made no counts of BrdU-labeled cells in other regions of the nasal cavity, we did note an obvious decrease in proliferation density with age in the organ of Masera (septal organ), a patch of olfactory epithelium located on the anterior ventral part of the septum. Similarly, in the vomeronasal organ, there was a clear decrease of proliferation density with age. In addition, the number of BrdU-labeled supporting cells in all olfactory epithelia declined with age.

Respiratory epithelium covers most of the nasal cavity and parts of the turbinates. Like the olfactory epithelium, it is repaired by

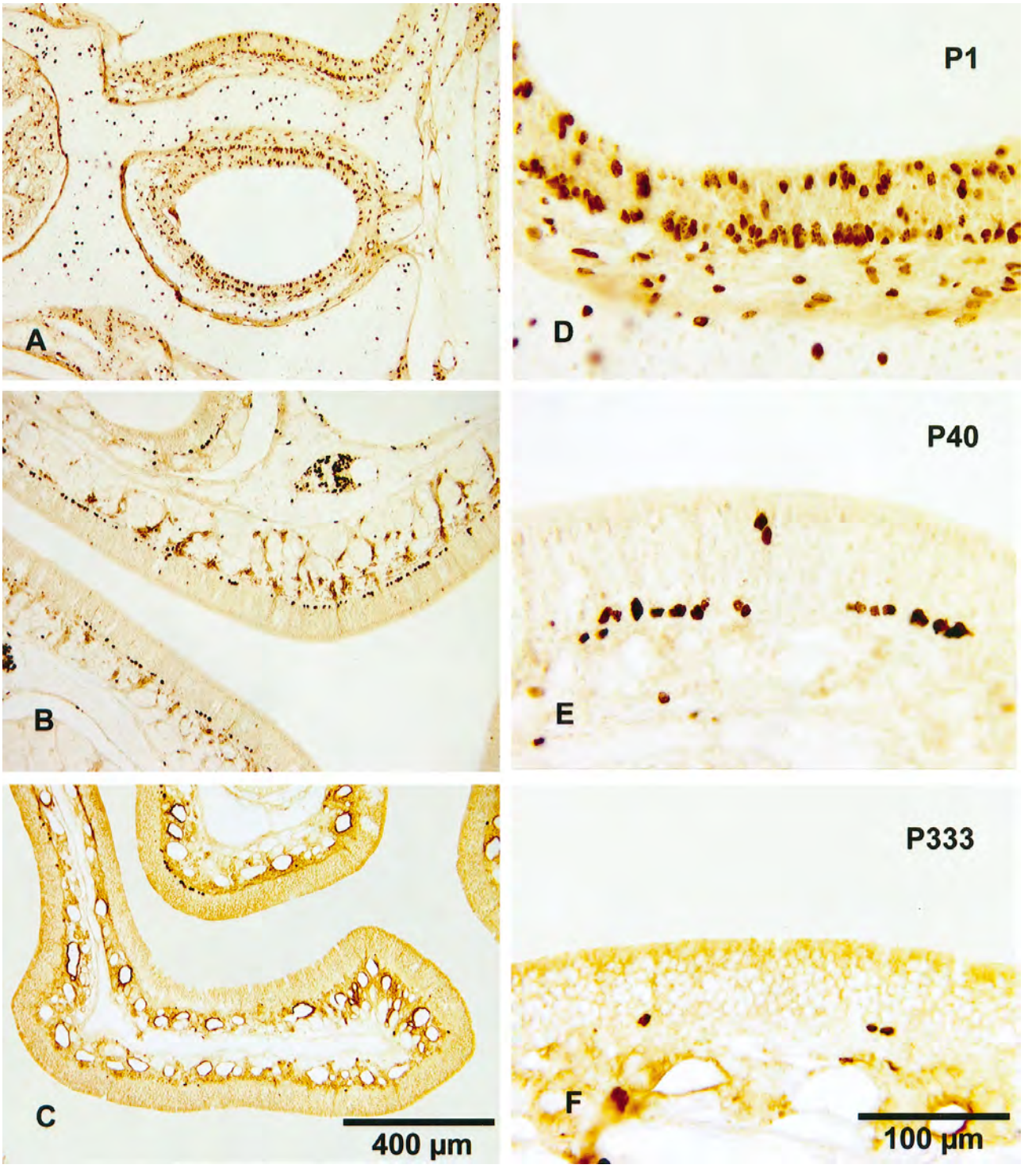

Figure 5. BrdU-labeled cells in the olfactory epithelium of animals at P1 (*A, D*), P40 (*B, E*), and P333 (*C, F*). The frequency of the labeled cells decreases dramatically with age. Whereas in newborns, the basal cells are so close together that they span nearly two rows (*D*), in P40 animals they form one line and/or are arranged in clusters (*E*). In P333 animals the clusters contain fewer cells and there are long spaces between clusters (*F*). The 400 μm marker in *C* also applies to *A* and *B*, and the 100 μm marker in *F* also applies to *D* and *E*.

replacement of the cells differentiating from daughter cells of mitotic basal cells. Here, too, it was readily apparent that the density of proliferating basal cells decreases with age, although again we did not make any counts.

Area of the olfactory epithelium

The area of the olfactory epithelium increases continuously with age in the range of ages we studied (Figs. 8, 15). This result confirms the data of others who measured area in postnatal rats

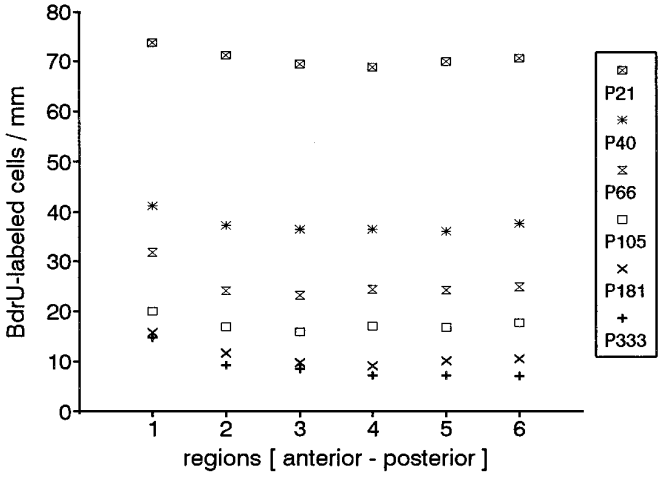

Figure 6. Proliferation density in the different regions ranging from anterior to posterior. There are no differences in the mean values among the regions (except region 1, see text). This is true for all age groups. Animals younger than P21 are not shown, because at those ages the regions are not exactly the same as in the other groups owing to the allometric growth of the turbinates. The SE values are omitted for clarity, but are included in Table 2.

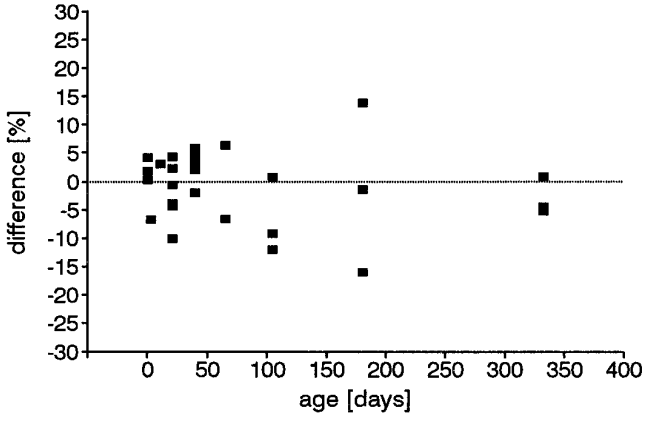

Figure 7. Proliferation density differences between the right and left sides of the olfactory epithelium in individual animals with the right side set at 100%. There is no trend or significant difference within any of the age groups.

from P1 to P90 (Meisami, 1989; Paternostro and Meisami, 1993) or used a different method and concentrated their measurements to the epithelium lining the septum (Hinds and McNelly, 1981; Apfelbach et al., 1991).

The profile of the area has a typical shape from anterior to posterior in the postweanling animals (Fig. 8; cf. Paternostro and Meisami, 1993). Moreover, the size of the anterior flat region of the curve increases with age so that the major part of the olfactory epithelium shifts posteriorly. This is consistent with the observation that the anterior region has the highest proliferative density. The length of epithelium begins to increase dramatically when the first ectoturbinate emerges and the second ectoturbinate acquires olfactory (rather than respiratory) epithelium. The shoulder of the curve preceding the apex is the region where the fourth endoturbinate appears, but is not yet covered with olfactory epithelium. More posteriorly, when olfactory epithelium covers this endoturbinate, the curve becomes steep again. The maximal length of olfactory epithelium is reached when all the turbinates are seen in

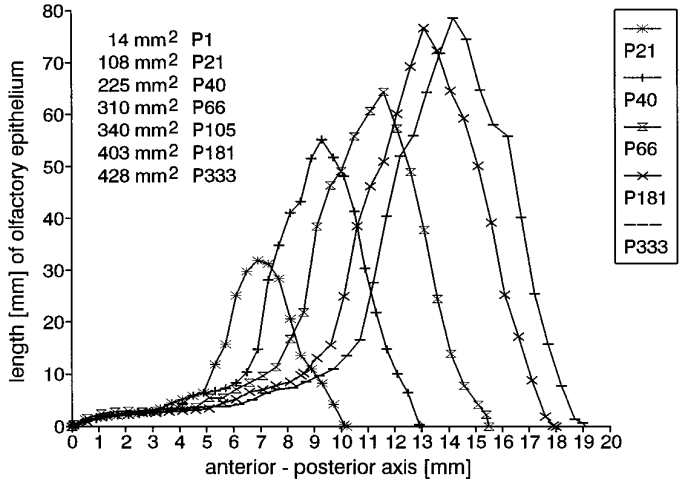

Figure 8. Typical distribution profiles of the extent of olfactory epithelium from anterior to posterior at different ages. Each *point* represents the length of olfactory epithelium on the right side measured in a frontal section at the respective level of the anterior–posterior axis. *Zero* represents the most rostral beginning of the olfactory epithelium. The area *under* each of these *curves* represents the area of the right olfactory sensory sheet in the respective animal. The area increases continuously during postnatal development. For clarity, the P1 and P105 curves are not shown, but the area values are given.

a single frontal section (region 3) before fusion of any turbinates. When the second endoturbinate begins to fuse (region 4), the length of olfactory epithelium in a section declines.

Olfactory epithelium thickness

Proliferation in the rat olfactory epithelium is required to provide new cells *both* for the age-related increase in the total olfactory surface area (Figs. 8, 15) and for the replacement of dying cells. Given that the total epithelial thickness is related to the number of neuronal cells (Mackay-Sim et al., 1988) and that both thickness and number are variable, it was deemed necessary to relate the proliferation density data to possible age-related changes in epithelial thickness and to the total number of various cell types within the epithelium.

The average thickness of the olfactory epithelium, measured from the basal lamina to the apical surface, showed age-dependent changes (Fig. 5). There was an increase in average thickness of 40% from birth to P40, whereas afterwards the average thickness decreased. The decrease continued in adults (Fig. 9). The probability is very high that the increase and decrease are real (Mann–Whitney U test, $p < 0.001$). No differences between males and females could be detected. The changes in thickness are not related to the decrease in number of proliferating cells with age.

It should be noted that although there was wide variability in the olfactory epithelium thickness, ranging in our study from 24 to 133 μm, one generalization can be made. Epithelium lining a convex structure was usually thicker than that lining a concave structure. The thickness of the epithelium lining the septum and turbinate edges (convex) was higher than the thickness in the vicinity where the turbinates were connected to the lateral wall (concave) or to other concave parts of the turbinates. It was clear from our observations that the decrease in epithelial thickness in the adult animals was real and not a result of any large increase in length of the concave or connecting parts with age, nor was it an artifact of oblique sectioning.

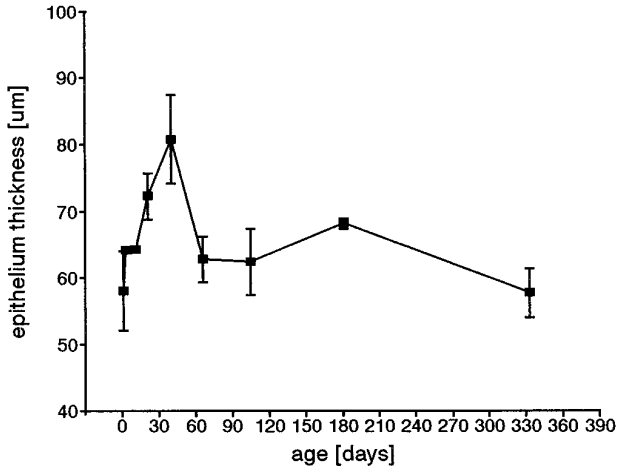

Figure 9. The epithelial thickness changes postnatally. There is an increase in thickness until P40, followed by a continuous decrease. Mean values and SD values are given for the different age groups.

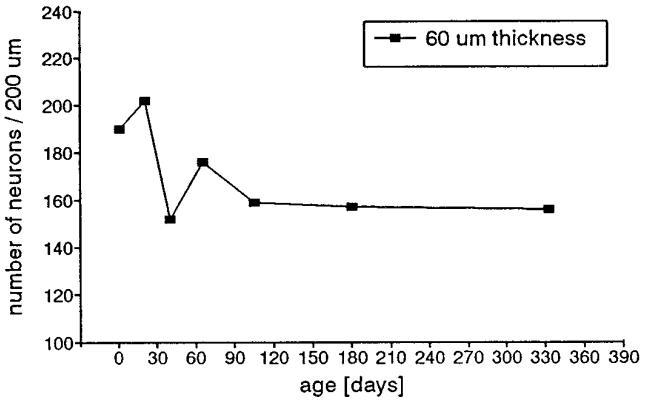

Figure 11. Number of nuclear profiles of neurons at different ages in a 200 μm length of olfactory epithelium and with a given thickness of 60 μm. The numbers were calculated for this thickness from the linear regression curves from the values in each age group. There is an increase in cell density from birth to P21 and then a decrease. The density stays nearly constant from P105.

Cell density

Because it is possible that the number of cells within a given length or volume of epithelium might not be constant, even if the epithelial thickness is constant, we estimated the number of nuclei of the three major epithelial cell types over a length of epithelium at different ages and different epithelium thicknesses. The data showed that in each age group there was a direct correlation between epithelial thickness and the number of *neurons* per unit length, thus confirming the data of others (cf. Mackay-Sim et al., 1988). Representative data are shown in Figure 10 for P21, and although the other data are not shown, this was true in all age groups. However, the neuronal density (number of neurons per unit length) in the different age groups was not always the same, even when the epithelial thickness was the same. We arbitrarily selected a thickness of 60 μm to evaluate neuronal density and by a linear regression calculation determined that in newborn animals (P1), there were 190 neurons in a 200 μm length of epithelium. This density increased to a peak of 202 at P21. In older age groups the density decreased and reached a plateau value of 160 from P105 on (Fig. 11). Thus, the change in density of neurons, even within a constant epithelial thickness, implies either a modification in their individual volumes, or perhaps a modification in the total volume occupied by the other cell types in the epithelium. However, the change in neuron density does not account for the dramatic decrease in proliferation density.

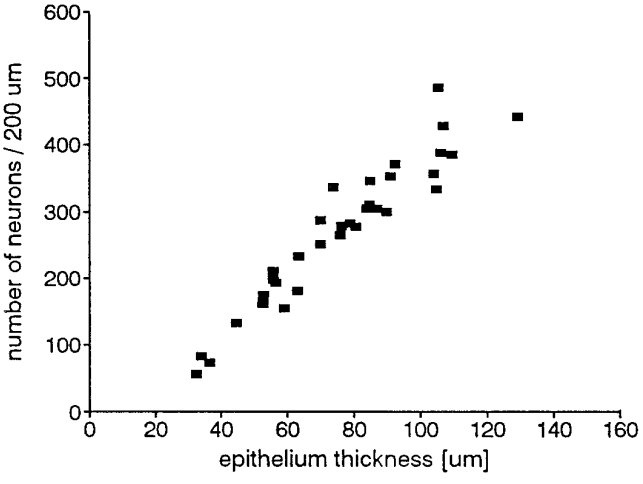

Figure 10. Number of nuclear profiles of neurons in a length of 200 μm olfactory epithelium at different thicknesses of the epithelium in P21 animals. There is a strong linear correlation between the number of neurons and the thickness of the epithelium. The number of cell nuclei increases with epithelial thickness. Each *point* represents the number of nuclei, the profiles for which lie within the 10 μm section, between the supporting cells and the basal cells.

Age-related change in ratio between number of proliferating cells and total number of neurons

It was of interest to determine whether the ratio between the number of basal cells incorporating BrdU and the total number of neurons per unit length changes with age, because this could have profound effects on the population dynamics. In other words, if this ratio remained the same at all ages, it would mean that there were no age-related changes in the growth rate of the total surface area and/or no difference in the rate at which cells are replaced. However, a decrease in this ratio between proliferating cell number and total neuron number might suggest, for example, that mature cells are living longer or that the rate of growth slows down, or the length of the cell cycle is increased.

In the assessment of this ratio, we did not limit our observations to a calculated value for a single thickness, as in Figure 11, but included all values at all thicknesses. We observed that the total number of neurons declined with age from an average high of ~1300/mm length at P21 to a low of <800/mm at P333 (Fig. 12). Accompanying the steep decline in total neuron number per unit length was an even greater reduction in the proportion of basal cells incorporating BrdU, from ~30% of the basal cells at P1, the percentage fell to <5% at P333 (Fig. 13). This is consistent with the possibility that the cell cycle becomes longer or that there are fewer basal cells in the cell cycle at any given time (i.e., more cells in G_0). Although the number of neurons/unit length decreased, the number of proliferating cells/unit length decreased even more; this was reflected in a decrease in the percentage of BrdU-labeled basal cells within the combined basal cell plus neuron population (Fig. 13). Thus, with age, the number of proliferating cells per unit length declined at a greater rate than the total number of neurons. This is consistent with the possibility that the neurons live longer.

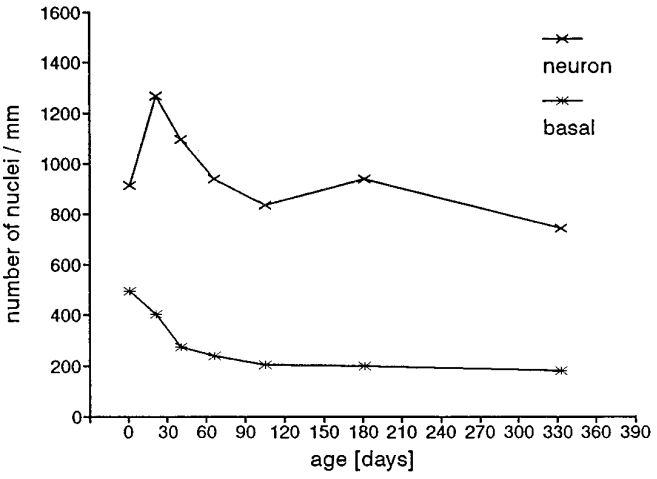

Figure 12. Number of neuronal profiles per millimeter length for the average thickness for each age group. The values in this graph were calculated from the linear regression curves for each age group. The number of neurons per millimeter length of the olfactory epithelium increases at P21 and then decreases almost continuously. This means that the total number of neurons in a given area would decline. The number of basal cells declines continuously from P1.

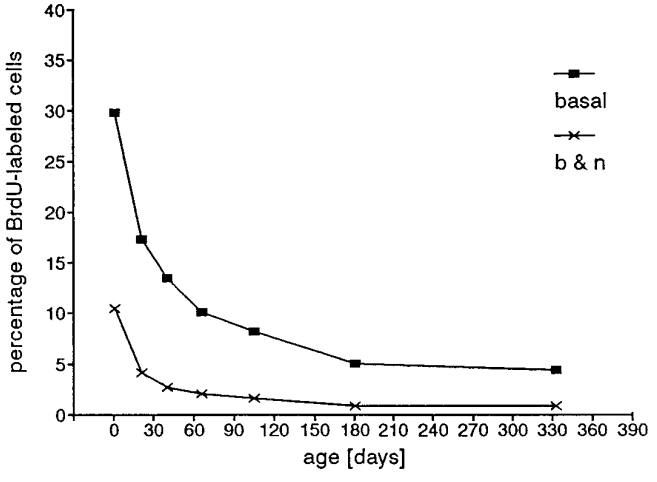

Figure 13. Percentage of BrdU-labeled cells in the basal cell compartment [number of BrdU-labeled cells divided by total number of basal cells (■)]. The *lower curve* (×) represents the calculated percentages of BrdU-labeled cells of the total number of neurons plus basal cells.

The ratio between the number of neurons and number of proliferating basal cells changed dramatically from 6:1 at P1 to 93:1 from P181 on (Fig. 14). In the rapidly growing olfactory epithelium of the P1 rat, the primary function of proliferating basal cells is very likely directed to increasing the total neuron population and surface area, whereas at P333, when surface area is growing much more slowly (Figs. 8, 15; Hinds and McNelly 1981), the major function of proliferation is replacement. Our results suggest that at P181, only a small population of proliferating basal cells, probably <5%, is directed to growth in area, whereas most of proliferation provides a pool for replacement.

DISCUSSION

Our results clearly show that age has an important influence on the number of BrdU-labeled basal cells/mm epithelial length in the rat olfactory epithelium. We observed a dramatic asymptotic

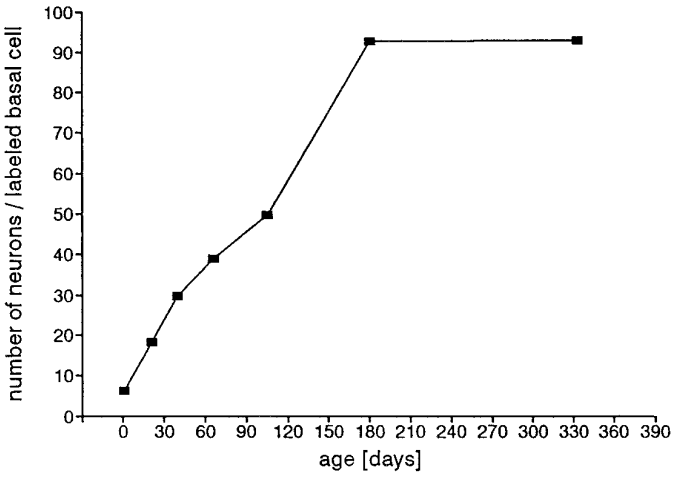

Figure 14. The ratio of the number of neurons to the number of BrdU-labeled basal cells changes significantly with age, from 6:1 in the neonate to 93:1 at P181 and P333.

decrease in proliferation density; the density in the 11-month-old rat was only 5% of that in the newborn.

Although there is a substantial difference in body size between adult male and female rats of the same age (Fig. 2) there was no sex-linked difference in proliferation density within any age group. This is consistent with the observations of others who showed that change in growth of the animal body size induced by thyroxin deficiency (Mackay-Sim and Beard, 1987; Paternostro and Meisami, 1993) or excess of growth hormones (Meisami et al., 1994) did not change the proliferation density in the olfactory epithelium. The importance of this observation lies in the possibility that a 200 gm male laboratory rat with no dietary restrictions can be as young as 36 d or as old as 56 d. Our data show that there would be a nearly twofold difference in proliferation density in rats at these two ages, although they might have the same body weight. Therefore, our data stress the importance of using age-matched controls in studies on proliferation density, rather than body weight-matched rats.

Patchy distribution of proliferating cells

We confirmed the data of others that proliferating basal cells appear in patches or clusters (Moulton et al., 1970; Graziadei and Monti Graziadei, 1979; Monti Graziadei and Graziadei, 1979; Suzuki and Takeda, 1993; Huard and Schwob, 1995). The number of clusters and labeled cells within a cluster was reduced with age. The patchy distribution of mitotic cells is consistent with the notion that the microenvironment is conducive for cell division, possibly because of the existence of a local stimulus that activates the cell cycle in several neighboring progenitor cells.

Another possible interpretation of why proliferative cells occur in clusters can be extrapolated from the data of Mackay-Sim and Kittel (1991b). They showed that the progenitor cell population of olfactory epithelium, the globose basal cells, can undergo two or more symmetric cell divisions before they leave the cell cycle and begin differentiation. In other words, a single globose basal cell divides symmetrically to give rise to two daughter cells, and each of these in turn could divide symmetrically to give rise to a total of four, etc. The clustering of BrdU-positive cells might then be explained by the existence of several neighboring progenitor cells with synchronized cell cycles. This would also be consistent with

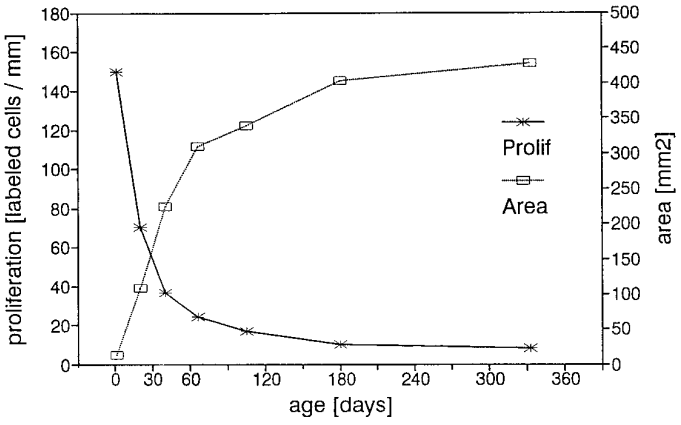

Figure 15. This graph shows the increase of the area of the olfactory sheet in postnatal development superimposed on the proliferation density (see Fig. 3). Area and proliferation density change reciprocally.

the notion stated above, namely, that a local stimulus could be responsible for initiating cell division in an individual patch.

Is the number of proliferating olfactory cells constant throughout life in the rat?

Our data and other studies on rat olfactory epithelium have shown that to the age of 18 months, there is an increase in the total area of the olfactory sheet associated with an increase in the total number of olfactory neurons (Hinds and McNelly, 1981; Meisami, 1989; Apfelbach et al., 1991; Paternostro and Meisami, 1993). The age-dependent decrease in proliferation density in the rat is reciprocally related to the continuous increase in the area covered by olfactory epithelium. We have used our area data to construct a curve (Fig. 15) depicting olfactory surface area and have superimposed this curve on a copy of the proliferation density curve from Figure 3. The total pool of BrdU-positive basal cells is distributed over a 25- to 30-fold larger epithelial surface area from the neonate to 11 months, resulting in a reduction of proliferation *density*. Although the density of labeled cells changes with age, the total *number* actually does not change a great deal, certainly by less than a factor of two in this study. In the very young rat, many or most of these proliferating cells probably contribute to the rapidly growing surface area of the olfactory epithelium as well as to the replacement of dying cells, whereas in later life when the rate of growth is considerably slower, their role may be more directed to cell replacement.

If the total number of proliferating cells remains nearly constant over the life of the animal, we must consider the possibility that some regulatory mechanism is acting to maintain this number. The number can be upregulated after bulbectomy (Costanzo, 1984; Schwartz Levey et al., 1991; Carr and Farbman, 1992), axotomy (cf. Nagahara, 1940; Costanzo and Graziadei, 1983; Nakamura, 1991; Suzuki and Takeda, 1991), direct destruction of the epithelium (Rehn et al., 1981; Cancalon, 1982; Schwob et al., 1995), or even by reducing the number of mitral cells in the bulb (Weiler and Farbman, 1996). The number can be downregulated after naris occlusion (Farbman et al., 1988; Cummings and Brunjes, 1994). We suggest that *dying cells may be the source of a local trigger that directly or indirectly promotes upregulation of mitotic rate, and that a downregulating signal might be derived from mature sensory neurons*. In older animals or in animals with unilateral naris closure, where the relative proportion of mature cells to total neurons is higher than in young adults, the balance would shift to a lower proliferation density.

The bulb contributes to mitotic regulation in the epithelium by maintaining the survival of neurons, presumably by delivering to them a trophic factor (Schwob et al., 1992). If this were true, there would have to be an increased supply of trophic substance to support survival of an increased number of neurons projecting to a constant number of mitral cells (Hinds and McNelly, 1981; Meisami, 1989). The convergence ratio of olfactory to mitral cells increases strikingly from 25:1 in the newborn rat to 250:1 at P25 (Meisami, 1989). In adult rabbits, the convergence may be as much as 6500:1 (Allison and Warwick, 1949). Morphometric measurements show that with age, mitral cells increase in size (Hinds and McNelly, 1981) and continue to produce GAP43, a protein known to be associated with neuron growth (Zhang et al., 1995). In addition, with increase in age, there is an increase in the number of periglomerular cells in the target region (Altman, 1969; Bayer, 1983; Alvarez-Buylla and Lois, 1995). The growth of mitral cells and/or the increased numbers of periglomerular cells in the target region provide more space for synapses, and the increased amount of target could be related to an increased production of trophic factor.

Do olfactory neurons in older animals live longer?

In mice raised in a filtered air environment, individual olfactory cells can live as long as 12 months (Hinds et al., 1984). Moreover, there is evidence for a greater density of mature neurons with advancing age in rats, if one uses as a sign of maturity the presence of a dendritic knob (Hinds and McNelly, 1981) or the relative proportion of mature neurons (OMP-positive cells) versus immature (B50/GAP43-positive) cells (Verhaagen et al., 1989). In some studies on cell dynamics during postnatal development, it was shown that the ratio between the number of dendritic knobs and the total number of neuronal cell bodies in a given volume of olfactory epithelium increases with age in ferrets, rats, and mice (Schmidt, 1989; Russ, 1989; Walker et al., 1990). These data are consistent with the notion that at least some olfactory neurons live longer in older animals. Other possible explanations for the higher proportion of mature cells and relatively fewer "almost mature" cells include the possibility that (1) some postmitotic cells die precociously, either for lack of trophic support from the bulb or for other reasons, and (2) an increase in the length of the cell cycle, i.e., fewer cells would likely be in the S-phase at the time of injection.

Our data do not permit one to make conclusions about the average life span of olfactory neurons or about the life span of individual cells. In fact, it may not even be useful to consider the *average* life span of the population of neurons, because its composition can change with age or unilateral naris occlusion. In both cases, the balance shifts in favor of mature neurons. It may be that life spans of olfactory neurons do not fit a Gaussian distribution but a distribution in which a high proportion of cells dies young, a high proportion lives for relatively long periods, and few cells live for intermediate periods. In contrast, younger animals have a significant population of "almost mature" (GAP43-positive) cells. Our data are consistent with the possibility that at least in older animals, when the growth rate has slowed, those olfactory neurons that reach maturity do live longer. More information is needed, however, to make intelligent estimates of life spans. For example, one must know how likely it is that a BrdU-labeled cell at any age will survive to maturity.

Conclusion

In summary, the significance of the age-related changes in the distribution of labeled olfactory progenitor cells may be attributable to any one or more of the following reasons: (1) there is less "demand" for replacement neurons in unperturbed older animals, because existing neurons live longer; (2) fewer new neurons are needed for an expanding olfactory area, because the rate of growth in older animals is much smaller; (3) in older rats there is a reduced number of symmetric divisions of progenitor cells (resulting in fewer cells per cluster); or (4) fewer cells are labeled because the cell cycle time is longer. At any age, however, when the demand for replacement is induced experimentally by massive cell death after olfactory bulbectomy or axotomy, the system is presumably able to respond by increasing the number of divisions in the progenitor cell population (Schwartz Levey et al., 1991; Suzuki and Takeda, 1991).

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ATTACHMENT G:

World Health Organization (WHO) (2001) WHO FOOD ADDITIVES SERIES 46: Furfuryl Alcohol and Related Substances. Joint FAO/WHO Expert Committee on Food Additives (JECFA).

WHO FOOD ADDITIVES SERIES 46:Furfuryl Alcohol and Related Substances

First draft prepared by
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Evaluation

Introduction

Estimated daily intake

Metabolic considerations

Application of the Procedure for the Safety Evaluation of Flavouring Agents

Consideration of combined intakes

Conclusions

Relevant background information

Explanation

Additional considerations on intake

Biological data

Absorption and transformation

Metabolism

Toxicological studies

Acute toxicity

Short-term studies of toxicity and long-term studies of toxicity and carcinogenicity

Genotoxicity

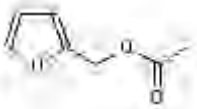
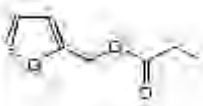
References

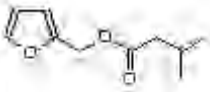
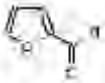
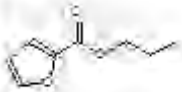
1. EVALUATION

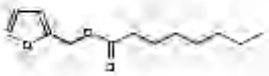
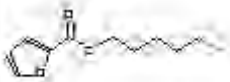
1.1 Introduction

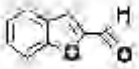
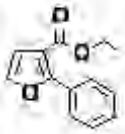
The Committee evaluated a group of 15 furfuryl derivatives (Table 1) by the Procedure for the Safety Evaluation of Flavouring agents. The group comprises the parent furfuryl alcohol, the corresponding aldehyde, furfural, five esters formed from furfuryl alcohol and simple aliphatic carboxylic acids, five esters formed from simple aliphatic alcohols and furoic acid, and three structurally related furfuryl derivatives (5-methylfurfural, 2-benzofurancarboxaldehyde, and 2-phenyl-3-carbethoxyfuran). These flavouring agents were grouped on the basis of the criterion that all are hydrolysed and/or metabolized to furoic acid or a substituted furoic acid.

Table 1. Summary of the results of the safety evaluation of furfuryl alcohol and 14 related flavouring agents[a]

Flavouring agent	No.	CAS No. and structure	*Step B3*[b] Does intake exceed the threshold for human intake?	*Step B4* Adequate NOEL for substance or structurally related substance?	Conclusion based on current intake
Structural class II					
Furfuryl alcohol[d]	451	98-00-0	No Europe: 210 USA: 24	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 10 000 times the estimated daily intake of furfuryl alcohol when used as a flavouring agent.	No safety concern
Furfuryl acetate[e]	739	623-17-6 	No Europe: 18 USA: 21	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 100 000 times the estimated daily intake of furfuryl acetate when used as a flavouring agent.	No safety concern
Furfuryl propionate[f]	740	623-19-8 	No Europe: 2 USA: 5	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 100 000 times the estimated daily intake of furfuryl propionate when used as a flavouring agent.	No safety concern
Furfuryl pentanoate[g]	741	36701-01-6	No Europe: 0.3 USA: 14	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural	No safety concern

				(Jonker, 2000), is > 100 000 times the estimated daily intake of furfuryl pentanoate when used as a flavouring agent.	
Furfuryl 3-methyl-butanoate[c]	743	13678-60-9 	No Europe: 0.03 USA: 1	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), the related substance, furfural (Jonker, 2000), furfuryl 3-methylbutanoate when used as a flavouring agent.	No safety concern
Furfural[d]	450	98-01-1 	No Europe: 520 USA: 460	Yes. The NOEL of 53 mg/kg bw per day (Jonker, 2000) is > 1000 times the estimated daily intake of furfural when used as a flavouring agent.	No safety concern
5-Methylfurfural	745	620-02-0	No Europe: 160 USA: 25	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 10 000 times the estimated daily intake of 5-methylfurfural when used as a flavouring agent.	No safety concern
Methyl-2-furoate[c]	746	611-13-2	No Europe: 35 USA: 37	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 10 000 times the estimated daily intake of methyl 2-furoate when used as a flavouring agent.	No safety concern
Propyl 2-furoate[c]	747	615-10-1 	No Europe: N/D USA: 0.1	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 10 million times	No safety concern

				the estimated daily intake of propyl 2-furoate when used as a flavouring agent.	
Structural class III					
Furfuryl octanoate[a]	742	39252-03-4 	No Europe: 0.01 USA: 6	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 1 000 000 times the estimated daily intake of furfuryl octanoate when used as a flavouring agent.	No safety concern
Amyl 2-furoate[c]	748	1334-82-2	No Europe: N/D USA: 0.1	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 10 million times the estimated daily intake of amyl 2-furoate when used as a flavouring agent.	No safety concern
Hexyl 2-furoate[c]	749	39251-86-0 	No Europe: N/D USA: 0.1	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 10 million times the estimated daily intake of hexyl 2-furoate when used as a flavouring agent.	No safety concern
Octyl 2-furoate[c]	750	39251-88-2	No Europe: 3 USA: 0.1	Yes. The NOEL of 53 mg/kg bw per day for the related substance, furfural (Jonker, 2000), is > 1 000 000 times the estimated daily intake of octyl 2-furoate when used as a flavouring agent.	No safety concern
2-Benzofurancarbox-aldehyde	751	4265-16-1	No Europe: N/D USA: 0.01	Yes. The NOEL of 25 mg/kg bw per day (Posternak et al., 1969) is > 100	No safety concern

				million times the estimated daily intake of 2-benzofuran-carboxaldehyde when used as a flavouring agent.	
2-Phenyl-3-carbeth-oxyfuran	752	50626-02-3 	No Europe: 0.01 USA: 2	Yes. The NOEL of 13 mg/kg bw per day (Postemak et al., 1969) is > 400 times the estimated daily intake of 2-phenyl-3-carbeth-oxyfuran when used as a flavouring substance.	No safety concern

CAS: Chemical Abstracts Service; N/D: no intake data reported

a Step 2. None of the substances in this group is expected to be metabolized to innocuous products.

b The thresholds for human intake for classes II and III are 540 µg/day and 90 µg/day, respectively. All intake values are expressed in µg/day.

c A group ADI of 0–0.5 mg/kg bw was established by the Committee as in present meeting for furfural, furfuryl alcohol, furfuryl acetate, furfuryl propionate, furfuryl pentanoate, furfuryl octanoate, furfuryl 3-methylbutanoate, methyl 2-furoate, propyl 2-furoate, butyl 2-furoate, hexyl 2-furoate, and amyl 2-furoate (see monograph addendum on furfural, p. 4).

The Committee has evaluated only one member of this group previously, namely furfural (No. 450). Furfural was considered by the Committee at its thirty-ninth and fifty-first meetings (Annex 1, references *101* and *137*), but no ADI was established. At its present meeting, the Committee established a group ADI of 0–0.5 mg/kg bw for furfural, furfuryl alcohol, and a number of derivatives of furfuryl alcohol and furoic acid on the basis of a NOEL of 53 mg/kg bw per day in a 13-week study in rats given furfural, with a safety factor of 100 (see monograph addendum on furfural, p. 3).

Seven of the 15 substances have been detected as natural components of foods (Maarse et al., 1994, 1996) including roasted coffee, beer, milk, roasted almonds, white bread, and whisky.

1.2 Estimated daily intake

The total annual volume of production of the 15 substances in this group was reported to be 6600 kg in Europe (International Organization of the Flavor Industry, 1995) and 4500 kg in the USA National Academy of Science, 1989; (Lucas et al., 1999). These values are equivalent to total daily per capita intakes of 940 µg in Europe and 590 µg in the USA. Furfural accounted for approximately 55% of the total per capita intake in Europe (520 µg/day) and 77% of that in the USA (460 µg/day). The annual volumes of use and estimated per capita intake of furfuryl alcohol and 14 related substances are shown in Table 2.

Table 2. Annual volume and estimated per capita intake of furfuryl alcohol and related substances in Europe and the United States[a]

Substance (No.)	Most recent annual volume (kg)[b]	Intake[b]		Consumption ratio[c]
		µg/day	µg/kg bw per day	
Furfuryl alcohol (451)			3800	

Europe	1438	210	3	
USA	186	24	0.4	
Furfuryl acetate (739)			85	
Europe	129	18	0.3	
USA	159	21	0.3	
Furfuryl propionate (740)			–	
Europe	14	2	0.03	
USA	37	5	0.1	
Furfuryl pentanoate (741)			–	
Europe	2	0.3	0.005	
USA	104	14	0.2	
Furfuryl octanoate (742)			–	
Europe	0.1	0.01	0.0002	
USA	45	6	0.1	
Furfuryl 3-methylbutanoate (743)			–	
Europe	0.2	0.03	0.0005	
USA	10	1	0.02	
Furfural (450)				66
Europe	3613	520	9	
USA	3470	460	8	
5-Methylfurfural (745)			750	
Europe	1120	160	3	
USA	191	25	0.4	
Methyl-2-furoate (746)			3	
Europe	243	35	0.6	
USA	279	37	0.6	
Propyl 2-furoate (747)			–	
Europe	N/D	N/D	N/D	

USA	0.5	0.1	0.001	
Amyl 2-furoate (748)			–	
Europe	N/D	N/D	N/D	
USA	0.5	0.1	0.001	
Hexyl 2-furoate (749)			–	
Europe	N/D	N/D	N/D	
USA	0.5	0.1	0.001	
Octyl 2-furoate (750)			–	
Europe	18	3	0.04	
USA	0.5	0.1	0.001	
2-Benzofurancarboxaldehyde (751)			–	
Europe	N/D	N/D	N/D	
USA	0.1	0.01	0.0002	
2-Phenyl-3-carbethoxy furan (752)			–	
Europe	0.1	0.01	0.0002	
USA	13	2	0.03	

N/D, no intake data reported; –, no quantitative information available on natural occurrence in foods

a From International Organization of the Flavour Industry (1995) and Lucas et al. (1999)

b Intake (µg/person per day) calculated as follows: [(annual volume, kg) × (1 × 10^9 µg/kg) / (population × survey correction factor × 365 days)], where population (10%, 'eaters only') = 32 × 10^6 for Europe and 26 × 10^6 for the USA. The correction factor = 0.6 for Europe and 0.8 for the USA, representing the assumption that only 60% and 80% of the annual volume of the flavour, respectively, was reported in the poundage surveys (International Organization of the Flavor Industry, 1995; Lucas et al., 1999). Intake (µg/kg bw per day) calculated as follows: [(µg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

c Calculated as follows: (annual consumption in food, kg)/(most recent reported volume as a flavouring agent, kg)

1.3 Metabolic considerations

Furfuryl esters are hydrolysed to furfuryl alcohol and the corresponding carboxylic acid. Furfuryl alcohol (No. 451) is subsequently oxidized to furfural (No. 450), which is then oxidized to 2-furoic acid. Furoate esters (Nos 746–750) are or are predicted to be hydrolysed directly to 2-furoic acid and the corresponding alcohol. Furoic acid forms a coenzyme A (CoA) thioester, which may be either metabolized to a glycine conjugate that is excreted in urine or condensed with acetyl CoA to form 2-furanacryloyl CoA, which is converted to a glycine conjugate and excreted in urine. The three remaining furfuryl derivatives (Nos 745, 751, and 752) are expected to follow similar metabolic pathways, i.e. ester hydrolysis, oxidation, and glycine conjugation, with side-chain oxidation (No. 745) or aromatic oxidation (Nos 751 and 752). In rodents, a minor pathway has been identified

which involves furan ring oxidation to produce carbon dioxide and as yet unidentified metabolites.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned substances, the Committee assigned nine of the 15 substances (Nos 450, 451, 739–741, 743, and 745–747) to structural class II (Cramer et al., 1978). Furfuryl propionate (No. 740) was assigned to this class because it is structurally closely related to furfuryl acetate (No. 739) and furfuryl pentanoate (No. 741). The remaining six substances (Nos 742 and 748–752) were assigned to structural class III.

Step 2. The data on the metabolism of individual members of the group were sufficient to draw conclusions about their probable metabolic fate. Most (Nos 450, 451, 739–743, and 746–750) are predicted to be metabolized to 2-furoic acid or a 2-furoic acid derivative, which is either conjugated with glycine and excreted in the urine or condensed with acetyl CoA and conjugated with glycine before excretion in the urine. Because of concern about the results of the toxicological studies on furfural in rodents, these substances cannot be predicted to be metabolized to innocuous products. The evaluation of all substances in this group therefore proceeded via the right-hand side of the scheme.

Step B3. The estimated daily per capita intakes of all 15 substances in this group are below the threshold of concern for the respective structural classes (i.e. 540 μg/day for structural class II and 90 μg/day for structural class III). Accordingly, the evaluation of all 15 substances in the group proceeded to step B4.

Step B4. For furfural, the NOEL of 53 mg/kg bw per day in a 13-week feeding study in rats (Jonker, 2000) provides an adequate margin of safety (> 1000 times) in relation to the known levels of intake of this substance. This NOEL is also appropriate for furfuryl alcohol (No. 451) and the structurally related substances furfuryl acetate (No. 739), furfuryl propionate (No. 740), furfuryl pentanoate (No. 741), furfuryl octanoate (No. 742), and furfuryl 3-methylbutanoate (No. 743), because all of these esters would be hydrolysed to furfuryl alcohol (No. 451) and then oxidized to furfural (No. 450). The NOEL for furfural is also appropriate for the esters of furoic acid, methyl 2-furoate (No. 746), propyl 2-furoate (No. 747), amyl 2-furoate (No. 748), hexyl 2-furoate (No. 749), and octyl 2-furoate (No. 750), which would be hydrolysed to furoic acid, the major metabolite of furfural. The NOEL for furfural is also appropriate for 5-methylfurfural (No. 745), which would participate in the same metabolic pathways and also undergoes alkyl oxidation. For 2-benzofurancarboxal-dehyde (No. 751), the NOEL of 25 mg/kg bw per day in a 90-day feeding study in rats (Posternak et al., 1969) provides an adequate margin of safety (> 1 000 000 times) in relation to the known levels of intake of this substance. For 2-phenyl-3-carbethoxyfuran (No. 752), the NOEL of 13 mg/kg bw per day in a 90-day feeding study in rats (Posternak et al., 1969) provides an adequate margin of safety (> 1000 times) in relation to the known levels of intake of this substance.

Table 1 summarizes the evaluation of furfuryl alcohol and 14 related substances used as flavouring agents.

1.5 Consideration of combined intakes

In the unlikely event that all foods containing the nine substances in structural class II (together with allyl 2-furoate evaluated previously by the Committee) were consumed concurrently on a daily basis, the estimated combined intake would exceed the threshold for human intake for class II. In the unlikely event that all foods containing the six substances in structural class III were consumed concurrently on a daily basis, the estimated combined intake would exceed the threshold for human intake for class III. Given the wide margin of safety between the level of intake and the NOEL for furfural and the fact that the available detoxication pathways (glycine conjugation or condensation followed by glycine conjugation) would not be saturated at the current levels of intake, the Committee concluded that the combined intake would not raise concern about safety.

1.6 Conclusions

On the basis of the predicted metabolism of these substances and data on their toxicity, the Committee concluded that consumption of furfuryl alcohol and the 14 related substances in this group at the current levels of intake would not raise concern for safety. In applying the Procedure, the Committee noted that all of the available data on toxicity are consistent with the results of the safety evaluation.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summaries key data relevant to the evaluation of the 15 flavouring substances in this group, which includes the parent alcohol, furfuryl alcohol, the corresponding aldehyde furfural, five esters formed from furfuryl alcohol and simple aliphatic carboxylic acids, five esters formed from simple aliphatic alcohols and furoic acid, and three structurally related furfuryl derivatives (5-methylfurfural, 2-benzofurancarboxyaldehyde, and 2-phenyl-3-carbethoxyfuran).

2.2 Additional considerations on intake

Seven of the 15 substances have been detected as natural components of foods (Maarse et al., 1994, 1996). Quantitative data on natural occurrence and consumption ratios have been reported for five substances in the group, and these indicate that they are consumed predominantly from traditional foods (i.e. consumption ratio >1) (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987; see Table 2).

The daily per capita intake of this group of flavouring agents is 940 µg/day in Europe (16 µg/kg bw per day) and 600 µg/day in the USA (10 µg/kg bw per day). Furfural accounted for approximately 55% of the total per capita intake in Europe (520 µg/day) and 77% in the USA (460 µg/day).

A recent study showed that furfural is formed in vivo during oxidative damage to DNA by hydroxylation of the C5' repair of deoxyribose (Dix et al., 1996). The oxidized deoxyribose fragment forms furfural, which may react subsequently with adenine bases to produce kinetin (N^6-furfuryladenine), which has been found in human DNA (Barciszewski et al., 1996, 1997a,b). The amount of furfural formed by this mechanism would be expected to be extremely small and the substance would be short-lived.

2.3 Biological data

Substances were considered to be members of this group on the basis that all furfuryl alcohol derivatives are hydrolysed and/or metabolized to furoic acid or a substituted furoic acid.

2.3.1 Absorption and transformation

Furfural is converted to furfuryl alcohol by enteric bacteria under both aerobic and anaerobic conditions (Boopathy et al., 1993). Both substances are therefore anticipated to be present in the gastrointestinal tract of animals given furfural. Furfuryl alcohol and furfural are rapidly absorbed in the gastrointestinal tract of rodents (Nomeir et al., 1992). At doses of 0.1–200 mg/kg bw, both are absorbed, metabolized, and excreted, primarily in the urine (Rice, 1972; Nomeir et al., 1992; Parkash & Caldwell, 1994). The results of the studies described below support this conclusion.

When [^{14}C]furfuryl alcohol at doses up to 27.5 mg/kg bw or [^{14}C]furfural at doses up to 12.5 mg/kg bw were administered to rats by gavage in corn oil, 83–89% of the radiolabel was excreted in the urine and 2–4% in the faeces, and 7% was exhaled as ^{14}C-carbon dioxide within 24 h. Residual radiolabel was distributed primarily to the liver and kidneys, the amount generally being proportional to the dose (Nomeir et al., 1992). Similar results were obtained by Parkash & Caldwell (1994) after oral administration of [^{14}C]furfural at doses up to 60 mg/kg bw in rats and up to 200 mg/kg bw in mice.

A similar pattern of absorption, distribution, and excretion was reported for alkyl-substituted furfural derivatives. In rats given [^{14}C]5-methylfurfural at doses up to 500 mg/kg bw by gavage, 70–82% of the administered dose was excreted in urine and 8–13% in faeces within 48 h. In mice given the same dose, a similar pattern of excretion were observed (Godfrey et al., 1999).

2.3.2 Metabolism

In general, furfuryl esters (furfuryl acetate, furfuryl propionate, furfuryl pentanoate, furfuryl octanoate, and furfuryl 3-methylbutanoate) are expected to be hydrolysed to furfuryl alcohol and the corresponding saturated aliphatic carboxylic acid, while furoate esters (methyl 2-furoate, propyl 2-furoate, amyl 2-furoate, hexyl 2-furoate, and octyl 2-furoate) are expected to be hydrolysed to 2-furoic acid and the corresponding saturated aliphatic alcohol. Hydrolysis is catalysed by classes of enzymes recognized as carboxylesterases or esterases (Heymann, 1980), the most important of which are the A-esterases. In mammals, A-esterases occur in most tissues of the body (Heymann, 1980; Anders, 1989), but they predominate in the hepatocytes (Heymann, 1980).

Evidence that hydrolysis may occur in the gastrointestinal tract comes from experiments by Grundschober (1977), who demonstrated that the structurally related substances isoamyl furylpropionate and ethyl furylpropionate were completely hydrolysed within 2 h by pancreatin. Similarly, Paul et al. (1948) showed that the glycine conjugate of furoic acid is the major metabolite in the urine of rats given furfuryl diacetate, furfuryl propionate, or methyl furylacrylate orally. This experiment demonstrates that these esters are hydrolysed in vivo to furfuryl alcohol, the metabolic precursor of furoic acid. The esters of furfuryl and furoate can be predicted to be hydrolysed to the parent alcohol and acid, respectively. Buck (2000) demonstrated rapid hydrolysis of furfuryl alcohol esters by preparations of rat liver, rat intestinal mucosa, and rat blood. While the rate of hydrolysis of these esters was slower in a human blood preparation, it was faster than those for other compounds with sterically hindered ester bonds, such as cinnamyl and linalyl esters.

After hydrolysis of the furfuryl esters, furfuryl alcohol is oxidized to furfural, which is subsequently oxidized to 2-furoic acid (Paul et al., 1948; ; Rice, 1972; Nomeir et al., 1992; Parkash & Caldwell, 1994). The major metabolic detoxication pathway for furoic acid involves formation of a CoA thioester, which is then either conjugated with glycine and excreted in the urine or condensed with acetyl CoA to form the CoA thioester of 2-furanacrylic acid. 2-Furanacryloyl CoA is also conjugated with glycine and excreted primarily in the urine (see Figure 1). A minor metabolic pathway has been reported in rodents whereby carbon dioxide is produced, presumably by furan-ring oxidation (Nomeir et al., 1992; Parkash & Caldwell, 1994).

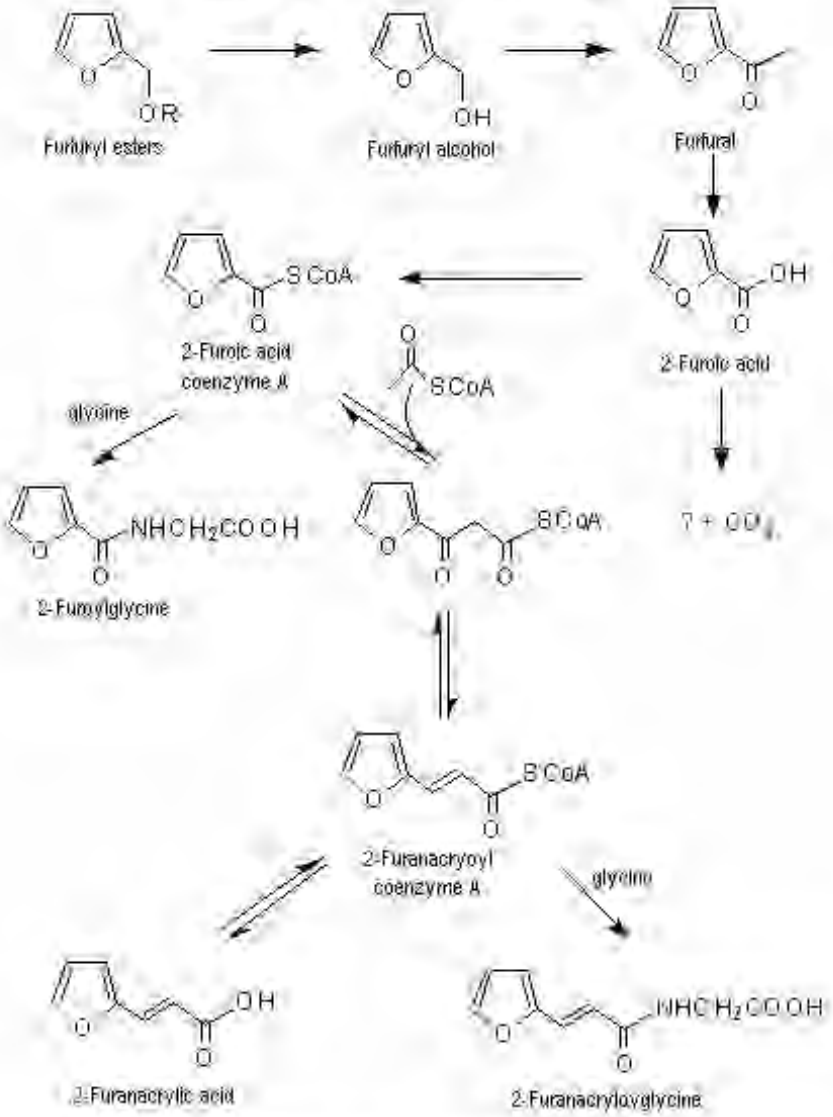


Figure 1. Metabolism of furfuryl alcohol in rats and mice

Adapted from Parkash & Caldwell (1994). R = aliphatic carboxylic acids; CoA, coenzyme A

A number of experiments provide evidence that these pathways exist. In rats, the glycine conjugate of furoic acid was detected in urine collected for 6 h after a single oral dose of 20 mg of furfuryl alcohol, furfural, furoic acid, furfuryl diacetate, furfuryl propionate, furanacylic acid, or methyl furanacrylate (Paul et al., 1948).

Furfuryl alcohol and furfural have been shown to participate in common pathways of detoxication in rodents. In groups of four male Fischer 344 rats, [^{14}C]furfuryl alcohol at oral doses up to 27.5 mg/kg bw or [^{14}C]furfural at 12.5 mg/kg bw were oxidized to furoic acid, which was excreted mainly in the urine, either as the glycine conjugate (73–80%) or free (1–6%). Furoic acid was also condensed with acetyl CoA and excreted in the urine as the glycine conjugate of furanacrylic acid (3–8%). In rats given furfural at 12.5 mg/kg bw, 7% was exhaled as carbon dioxide. Over the dose ranges studied, the extent of metabolism, the relative amounts of metabolites, and the rates of excretion were linear (Nomeir et al., 1992).

Single oral doses of [^{14}C]furfural (0.1, 10, or 60 mg/kg bw) given to male and female Fischer 344 rats or [^{14}C]furfural (1, 20, or 200 mg /kg bw) given to male and female CD-1 mice were metabolized to the glycine conjugates of furoic acid (76–84% in rats and 65–89% in mice) and furanacrylic acid (16–24% in rats and 10–17% in mice) within 24 h. Male mice at the high dose expired < 5% of the radiolabel as [^{14}C]carbon dioxide, but expired radiolabel was not measured in mice at other doses. The absorption, tissue distribution, extent of metabolism, relative amounts of metabolites, including carbon dioxide, and the rates of excretion in rodents

were linear over the range of doses investigated (0.1–200 mg/kg bw) (Parkash & Caldwell, 1994).

The condensation of furoic acid with acetyl CoA to yield furanacrylic acid appears to be a dynamic equilibrium favouring the CoA thioester of furoic acid (Parkash & Caldwell, 1994), as suggested by the observation that furoic acid was excreted in the urine of dogs given furanacrylic acid (Friedmann, 1911). An analogous equilibrium was established between other aromatic carboxylic acids (e.g. benzoic acid and cinnamic acid) (Nutley et al., 1994). Excretion of free furoic acid and furanacrylic acid in animals given higher doses suggests that the capacity for glycine conjugation may be limited, probably by the supply of endogenous glycine (Gregus et al., 1993).

In rats, the metabolic fate of 5-methylfurfural is similar to that of furfural. Two urinary metabolites were identified when rats were given an oral dose of an aqueous solution containing 5-methylfurfural at 80, 120, or 160 mg/kg bw. The principal metabolite was the glycine conjugate of 5-methylfuroic acid (> 40%); 5-methylfuryl methyl ketone was a minor metabolite (6.6–7.8%). 5-Methylfuryl methyl ketone was also a urinary metabolite of 5-methylfuroic acid, suggesting that the ketone is formed directly from the acid (Liebert, 1988). In a manner similar to the formation of furan-acrylic acid from furfural, the CoA thioester of 5-methylfuroic acid condenses with acetyl CoA to form an *alpha*-ketoester which sequentially undergoes hydrolysis and decarboxylation to form 5-methylfuryl methyl ketone. 5-Methylfuryl methyl ketone may be excreted in the urine or reduced to the corresponding alcohol before excretion, in a manner similar to that of structurally related furyl ketones (Boyd et al., 1975).

Groups of male Fischer 344 rats and male $B6C3F_1$ mice were given [^{14}C]-5-methylfurfural at a dose of 5, 10, 100, or 500 mg/kg bw by oral gavage. 5-Hydroxymethyl-2-furoic acid was the major urinary metabolite in both species and accounted for 77–90% of the recovered radiolabel at each dose. The quantity of the glycine conjugate *N*-(5-hydroxymethyl-2-furoyl)-glycine excreted by rats was inversely proportional to the dose, possibly due to glycine depletion at higher doses. 2,5-Furandicarboxylic acid accounted for only 2–4% of the recovered radiolabel in rats and 4–6% in mice (Godfrey et al., 1999). Similarly, 5-hydroxymethylfurfural administered to rats as single oral doses of 0.08–330 mg/kg bw was excreted mainly as 5-hydroxymethyl-2-furoic acid and to a lesser extent as *N*-(5-hydroxymethyl-2-furoyl)glycine (Germond et al., 1987).

The formation of labelled carbon dioxide in rats and mice presumably occurs via decarboxylation of furoic acid. No evidence of decarboxylation has been observed in humans (Flek & Sedivec, 1978). Since heteroaromatic and aromatic carboxylic acids do not normally decarboxylate in vivo (Caldwell, 1982), it can be assumed that oxidation of furoic acid in rodents precedes the loss of carbon dioxide. Epoxidation (Ramsdell & Eaton, 1990) or hydroxylation (Ravindranath & Boyd, 1985; Koenig & Andreesen, 1990) of the furan ring may yield reactive intermediates such as furfural-2,3-epoxide, acetylacrolein or *alpha*-ketoglutaric acid, which are readily decarboxylated. Biochemical changes in the lungs and livers of animals exposed to furfural indicate that ring oxidation may be catalysed by a cytochrome P450b isoenzyme to yield a reactive intermediate such as an epoxide or acylacrolein, which is subsequently conjugated with glutathione (Gupta et al., 1991; Mishra et al., 1991). There is no evidence of ring oxidation in humans at low levels of exposure. Essentially all the furfural absorbed (2 mg/kg bw) by inhalation in a study in humans was excreted in the urine as furoylglycine and furanacrylic acid, and no free furoic acid was found (Flek & Sedivec, 1978).

In summary, the component linear saturated alcohols and carboxylic acids (C1–C8) formed by ester hydrolysis of furfuryl esters and furoic acid esters participate in fatty acid *beta*-oxidation and the citric acid cycle or in the C1 tetrahydrofolate pathway, eventually to yield carbon dioxide and water (Voet & Voet, 1990). The available data indicate that most of the 15 furfuryl derivatives would be hydrolysed and/or oxidized to yield furoic acid. In humans, furoic acid would be efficiently excreted as either 2-furoylglycine or 2-furanacryloylglycine (2-furanacryluric acid).

2.3.2 Toxicological studies

2.3.2.1 Acute toxicity

LD_{50} values after oral administration are available for four of the 15 furfuryl derivatives. The values reported in rodents range from 50 mg/kg bw for furfural (Fassett, 1963) to 2200 mg/kg bw for 5-methylfurfural (Moreno, 1978). Most of the values fall between 100 and 250 mg/kg bw (Boyland, 1940; Gajewski & Alsdorf, 1949; Haag et al., 1959; Jenner et al., 1964). In rodents, signs of acute toxicity are seen mainly in the liver, and high doses are associated with hepatic cirrhosis accompanied by sporadic eosinophilic degeneration of hepatic cells. Nuclear pyknosis, eosinophilic necrosis, and increased hepatocyte mitosis have also been reported (Shimizu & Kanisawa, 1986). The changes observed in cytosolic and mitochondrial enzyme activities are indicative of increased hepatocellular catabolism (Jonek et al., 1975; Kaminska, 1977).

2.3.2.2 Short-term and long-term studies of toxicity and carcinogenicity

The results of short-term and long-term studies of the toxicity of the substances in this group are shown in Table 3. Details of the studies that were critical to evaluating the safety of furfuryl alcohol, furfural, 2-benzofurancarboxaldehyde, and 2-phenyl-3-carbethoxy furan are given below.

Table 3. Results of short-term studies of toxicity and long-term studies of toxicity and carcinogenicityon furfuryl alcohol and related substances

No.	Substance	Species, sex	No. test groups[a]/ no. per group[b]	Route	Duration	NOEL (mg/kg bw per day)	Reference
738	Furfuryl alcohol	Mice; M, F	5/5	Inhalation	16 days	Not identified	National Toxicology Program (1999)
738	Furfuryl alcohol	Mice; M, F	6/10	Inhalation	14 weeks	Not identified	National Toxicology Program (1999)
738	Furfuryl alcohol	Mice; M, F	4/50	Inhalation	105 weeks	Not identified	National Toxicology Program (1999)
738	Furfuryl alcohol	Rats; M, F	5/5	Inhalation	16 days	Not identified	National Toxicology Program (1999)
738	Furfuryl alcohol	Rats; M, F	6/10	Inhalation	14 weeks	Not identified	National Toxicology Program (1999)
738	Furfuryl alcohol	Rats; M, F	4/50	Inhalation	105 weeks	Not identified	National Toxicology Program (1999)
744	Furfural	Mice; M, F	5/10	Gavage	16 days	Not identified	National Toxicology Program (1990)
744	Furfural	Mice; M, F	5/20	Gavage	13 weeks	Not identified	National Toxicology Program (1990)
744	Furfural	Mice; M, F	3/100	Gavage	2 years	Not identified	National Toxicology

							Program (1990)
744	Furfural	Rats; M, F	5/10	Gavage	16 days	Not identified	National Toxicology Program (1990)
744	Furfural	Rats; ,M, F	5/20	Gavage	13 weeks	Not identified	National Toxicology Program (1990)
744	Furfural	Rats; M, F	2/100	Gavage	2 years	Not identified	National Toxicology Program (1990)
744	Furfural	Rats; M, F	5/5	Diet	14 days	120	Jonker (1998)
744	Furfural	Rats; M, F	4/20	Diet[c]	13 weeks	60	Jonker (2000)
744	Furfural	Hamsters; M, F	1/70	Intratracheal	36 weeks	Not identified	Feron (1972)
751	2-Benzofuran carboxaldehyde	Rats; M, F	1/32	Diet	90	25 (M)[d] 27 (F)[d]	Posternak et al. (1969)
752	2-Phenyl-3-carbethoxy furan	Rats; M, F	1/30	Diet	90	13	Posternak et al. (1969)

M, male; F, female

a Total number of test groups does not include control animals.

b Total number per test group includes both male and female animals.

c Furfural was administered in the food in microencapsulated form.

d Single dose

The component aliphatic acyclic alcohols and carboxylic acids formed by hydrolysis of the furfuryl and furoic acid esters are acetic acid, propionic acid, valeric acid, octanoic acid, methanol, propanol, pentyl alcohol, hexyl alcohol, and octyl alcohol. The alcohols are readily oxidized to the corresponding acids, which are endogenous to humans. The results of short-term and long-term studies of oral administration of seven of these simple aliphatic acids indicate no evidence of toxicity at an intake of 400 mg/kg bw per day or more (Butterworth et al., 1978; Bueld & Netter, 1993).

Furfuryl alcohol (No. 738)

No short- or long-term studies on the toxicity of furfuryl alcohol administered orally were available, but the substance has been administered by inhalation to both mice and rats. Since furfuryl derivatives are expected to be absorbed rapidly (Flek & Sedevic, 1978), regardless of the route of administration, these studies are described here; however, the NOELs reported by the authors of these studies are not relevant to ingestion of furfural alcohol in the diet, since the toxic effects observed were confined mainly to the olfactory system and respiratory tract (Feron & Kruysse, 1978; Mellick et al., 1991; National Toxicology Program, 1999). While these studies are of limited value for assessing the potential toxicity of oral exposure to furfuryl alcohol, the findings may provide some indication of potential long-term toxicity.

Groups of 10 $B6C3F_1$ mice of each sex were exposed to furfuryl alcohol by inhalation at a concentration of 0, 2, 4, 6, 8, 16, or 32 ppm for 6 h/day, 5 days/week, for 14 weeks. No deaths were reported. The hearts of males exposed to 32 ppm weighed significantly less than those of controls. Dose-dependent increases in the incidences of non-neoplastic lesions of the respiratory, olfactory, and transitional epithelium were observed in all treated groups (National Toxicology Program, 1999).

Groups of 50 $B6C3F_1$ mice of each sex were exposed to furfuryl alcohol by inhalation at a concentration of 0, 2, 4, 8, or 32 ppm for 6 h/day, 5 days/week, for 105 weeks. The body weights were measured weekly for the first 12 weeks, monthly until week 91, and every 2 weeks thereafter. Clinical signs of toxicity were recorded monthly for the first 91 weeks and every 2 weeks thereafter. Complete necropsies and microscopic examinations were performed on all test and control animals. The survival rates for all treated groups were comparable to those of chamber controls. The mean body weights of exposed females were significantly reduced during the second year of exposure, but the body weights of males were similar to those of controls throughout the study. Male mice exposed to 32 ppm had a significantly increased incidence of renal tubular degeneration (control, 0/50; 32 ppm, 48/50) and a significantly increased combined incidence of renal tubular adenomas and carcinomas (control, 0/50; 32 ppm, 5/50). The incidences of a variety of non-neoplastic lesions of the nose were significantly increased in all treated groups with respect to controls. Corneal degeneration was significantly more frequent in females exposed to 32 ppm furfuryl alcohol than in control mice (control, 3/49; 32 ppm, 26/50) (National Toxicology Program, 1999).

Groups of 10 Fischer 344/N rats of each sex were exposed to furfuryl alcohol by inhalation at a concentration of 0, 2, 4, 6, 8, 16, or 32 ppm for 6 h/day, 5 days/week, for 14 weeks. No deaths were reported. The body weights of females exposed to 32 ppm of furfuryl alcohol were significantly reduced at the end of the study when compared with chamber controls. Dose-dependent increases in the incidences of non-neoplastic lesions of the respiratory, olfactory, and transitional epithelium were observed in all treated groups (National Toxicology Program, 1999).

Groups of 50 Fischer 344/N rats of each sex were exposed to furfuryl alcohol by inhalation at a concentration of 0, 2, 4, 8, or 32 ppm for 6 h/day, 5 days/week, for 105 weeks. The observations and examinations followed the same protocol used in the corresponding study in mice, described above. All males exposed to 32 ppm had died by week 99, but the percentage survival of the other groups was comparable to that of the chamber control group. The terminal body weights of males exposed to 32 ppm were significantly lower than those of controls. No treatment-related clinical effects were observed. Males at the highest dose had significantly increased incidences of mineralization and renal tubular hyperplasia, as revealed in step-section analysis of kidney tissues. A dose-related increase in the severity of renal nephropathy was reported in exposed males and females. Significantly increased incidences of parathyroid gland hyperplasia (control, 10/50; 32 ppm, 25/50) and fibrous osteodys-trophy in the bones (control, 2/50; 32 ppm, 34/50) were reported in males at the highest dose (National Toxicology Program, 1999).

In a 13-week study, groups of 10 Fischer 344 rats of each sex were exposed by inhalation to atmospheres containing furfuryl alcohol at a concentration of 0, 2, 4, 8, 16, or 32 ppm. No treatment-related effects were observed on survival or organ weights. The final body weights of animals of each sex at 32 ppm were slightly reduced. Sperm morphology and vaginal cytology in animals at 0, 2, 8, and 32 ppm did not reflect significant reproductive toxicity, except for a slight increase in the number of spermatid heads in males at 8 and 32 ppm. Histological evaluation revealed lesions typical of chronic irritation of the nasal cavity and respiratory epithelia. These changes were minimal in animals at 2 and 4 ppm. No hepatic effects were reported (Mellick et al., 1991).

Furfural (No. 744)

A 16-day study in $B6C3F_1$ mice given furfural at a dose of 0, 25, 50, 100, 200, or 400 mg/kg bw per day, a 13-week study in $B6C3F_1$ mice at doses of 0, 75, 150, 300, 600, or 1200 mg/kg bw per day, and a 2-year study in $B6C3F_1$ mice at doses of 0, 50, 100, or 175 mg /kg bw per day, all by gavage in corn oil (National Toxicology Program, 1990) were evaluated by the Committee at its fifty-first meeting (Annex 1, reference *137*).

Two sets of studies of orally administered furfural have been conducted in Fischer 344N rats: one by the National Toxicology Program (1990) in which furfural was administered by gavage in corn oil, and one conducted by Jonker (1998, 2000) with microencapsulated furfural in the diet. A 16-day study in rats given furfural at a dose of 0, 15, 30, 60, 120, or 240 mg/kg bw per day, a 13-week study in rats at a dose of 0, 11, 22, 45, 90, or 180 mg/kg bw per day, and a 2-year study in rats at a dose of 0, 30, or 60 mg/kg bw per day, all by gavage in corn oil (National Toxicology Program, 1990) were evaluated by the Committee at its fifty-first meeting (Annex 1, reference *137*). A 14-day study in rats at doses of 0, 30, 60, 90, 120, or 180 mg/kg bw per day and a 13-week study in rats at doses of 0, 30, 60, 90, or 180 mg/kg bw per day, both with microencapsulated furfural, were evaluated by the Committee at the present meeting and are summarized in the monograph addendum on furfural.

Syrian golden hamsters were given 0.2 ml of 1.5% furfural in physiological saline (about 25 mg/kg bw), 0.5% benzo[*a*]pyrene (8.3 mg/kg bw), or 1.5% furfural plus 0.5% benzo[*a*]pyrene intratracheally once per week for 36 weeks. No respiratory tract or peritracheal tumours were found. No tumorigenic effects were observed after the administration of furfural alone (Feron, 1972).

The effect of furfural on the carcinogenic potential of benzo[*a*]pyrene and *N*-nitrosodiethylamine was evaluated in male and female hamsters exposed to atmospheres containing 400 ppm (1550 mg/m^3) of furfural for 7 h/day, 5 days/week, for 9 weeks, 330 ppm (1280 mg/m^3) for the next 11 weeks, and 250 ppm (970 mg/m^3) for an additional 32 weeks. The respiratory effects included atrophy and downward growth of the olfactory epithelium, degenerative changes in Bowman's glands, and the appearance of cyst-like structures in the lamina propria beneath the olfactory epithelium. Although exposure to furfural was irritating to the olfactory epithelium, the treatment was not toxic to the liver (Feron & Kruysse, 1978).

2-Benzofurancarboxaldehyde (No. 751)

Groups of 16 Charles River rats of each sex were maintained on basal diets or diets containing 2-benzofurancarboxaldehyde at a concentration calculated to result in an average daily intake of 25 mg/kg bw for males and 27 mg/kg bw for females, for 90 days. Weekly measurements of body weight and food intake showed no significant differences between treated and control animals. Clinical chemistry, haematology, and urine analyses conducted during weeks 7 and 13 revealed normal values. The organ weights at necropsy, gross examination, and histology showed no treatment-related effects. The NOEL was 25 mg/kg bw per day (Posternak et al., 1969).

2-Phenyl-3-carbethoxy furan (No. 752)

Groups of 15 Wistar rats of each sex were maintained on basal diets containing 2-phenyl-3-carbethoxy furan at a concentration calculated to result in an average daily intake of 13 mg/kg bw, for 90 days. Weekly measurements of body weight and food intake showed no significant differences between treated and control animals. Clinical chemistry, haematology, and urine analyses conducted during weeks 7 and 13 revealed normal values. The organ weights at necropsy, gross examination, and histology showed no treatment-related effects. The NOEL was 13 mg/kg bw per day (Posternak et al., 1969).

2.3.2.3 Genotoxicity

The results of studies of the genotoxicity of furfurylalcohol and related substances are shown in Table 4.

Table 4. Studies of the genotoxicity of furfuryl alcohols and related substances

No.	Substance	End-point	Test object	Concentration	Result[a]	Reference
738	Furfuryl alcohol	Reverse mutation	S. typhimurium TA98, TA100, TA1535, TA1537	294 µg/plate	Negative[a,b]	Florin et al. (1980)
738	Furfuryl alcohol	Reverse mutation	S. typhimurium TA98, TA100, TA1535	≤ 10 000 µg/plate	Negative[a,b]	Mortelmans et al. (1986)
738	Furfuryl alcohol	Reverse mutation	S. typhimurium TA100	2500–12 500µg/ml	Negative[a,b]	Stich et al. (1981a)
738	Furfuryl alcohol	Reverse mutation	S. typhimurium TA98, TA100, TA102	≤ 198 000 µg/plate	Negative[a,b]	Aeschbacher et al. (1989)
738	Furfuryl alcohol	Reverse mutation	S. typhimurium TA98, TA100	81–323 µg/plate	Negative[a,b]	Shinohara et al. (1986)
738	Furfuryl alcohol	Reverse mutation	S. typhimurium TA1535, TA100, TA1537 (modified assay)	200 000 µg/ml	Positive[a,b]	McGregor et al. (1981)
738	Furfuryl alcohol	DNA repair and H17 (rec+)	B. subtilis M45 (rec–) µg/disc	2000–20 000	Positive[a,b]	Shinohara et al. (1986)
738	Furfuryl alcohol	Sister chromatid exchange	Chinese hamster ovary cells	245 µg/ml	Positive[a,b]	Stich et al. (1981b)
738	Furfuryl alcohol	Sister chomatid exchange	Human lymphocytes	≤ 196 µg/ml	Negative	Jansson et al. (1986)

738	Furfuryl alcohol	Siser chromatid exchange	Human lymphocytes	≤ 970 µg/ml	Negative	Gomez-Arroyo & Souza (1985)
738	Furfuryl alcohol	Chromosomal aberration	Chinese hamster ovary cells	2000 µg/ml	Positive	Stich et al. (1981b)
738	Furfuryl alcohol	Gene conversion	S. cerevisiae strain D7	13 500–16 000 µg/ml	Positive[a]	Stich et al. (1981a)
738	Furfuryl alcohol	Sex-linked recessive l lethal mutation	D. melanogaster	≤ 6500 ppm by injection	Negative	Rodriguez-Arnaiz et al. (1989)
738	Furfuryl alcohol	Sister chromatid exchange	Adult human lymphocytes	32 300 mg/m^3 in occupational atmosphere	Negative	Gomez-Arroyo & Souza (1985)
738	Furfuryl alcohol	Sister chomatid exchange	Adult human lymphocytes	32 300 mg/m^3 in occupational atmosphere	Negative	Gomez-Arroyo & Souza (1985)
738	Furfuryl alcohol	Chromosomal aberration	Mouse bone-marrow cells drinking-water	0.5 mg/kg bw in	Negative	Sujatha & Subramanyam (1994)
				1–2 mg/kg bw in drinking-water	Positive	
738	Furfuryl acetate	Reverse mutation	S. typhimurium TA1535, TA98, TA100	33–666 µg/plate	Positive[a,b]	Mortelmans et al. (1986)
744	Furfural	Reverse mutation	S. typhimurium TA 1535, TA100, TA1537, TA1538, TA98	0.1–1000 µg/ml	Negative[a,b]	McMahon et al. (1979)
744	Furfural	Reverse mutation	S. typhimurium TA100, TA98, TA1535	≤ 3460 µg/plate	Negative[a,b]	Loquet et al. (1981)
				5766 µg/plate	Positive[a] (weakly)	
744	Furfural	Reverse mutation	S. typhimurium TA100, TA98, TA102	≤ 115 320 µg/plate	Negative[a,b]	Aeschbacher et al. (1989)
744	Furfural	Reverse mutation	S. typhimurium TA100, TA98	15–63 µg/plate	Negative[a,b]	Shinohara et al. (1986)
744	Furfural	Reverse mutation	S. typhimurium TA104	5–500 µg/plate	Positive[b]	Shane et al. (1988)
744	Furfural	Reverse mutation	S. typhimurium TA100, TA102	5–500 µg/plate	Negative[b]	Shane et al. (1988)

744	Furfural	Reverse mutation	S. typhimurium TA104, TA102	96 µg/plate	Negative	Marnett et al. (1985)
744	Furfural	Reverse mutation	S. typhimurium TA98, TA100, TA1535	≤ 6667 µg/plate	Negative[a,b]	Mortelmans et al. (1986)
744	Furfural	Reverse mutation	S. typhimurium TA98, TA100	≤ 1000 µg	Negative[a]	Osawa & Namiki (1982)
744	Furfural	Reverse mutation	S. typhimurium TA98, TA100, TA1535, TA1537	33– 6666 µg/plate	Negative[a,b] Equivocal in TA100[a]	National Toxicology Program (1990)
744	Furfural	Reverse mutation	S. typhimurium TA100	8000 µg/plate	Positive[a,b]	Zdzienicka et al. (1978)
744	Furfural	Reverse mutation	S. typhimurium TA98	8000 µg/plate	Negative[a,b]	Zdzienicka et al. (1978)
744	Furfural	Reverse mutation	S. typhimurium TA100, TA102	100–10 000 µg/plate	Negative[a]	Dillon et al. (1998)
744	Furfural	Reverse mutation	S. typhimurium TA104	100–10 000 µg/plate	Equivocal[a]	Dillon et al. (1998)
744	Furfural	Reverse mutation	S. typhimurium TA102, TA104	100–10 000 µg/plate	Negative[b]	Dillon et al. (1998)
744	Furfural	Reverse mutation	S. typhimurium TA100	100–10 000 µg/plate	Equivocal[b]	Dillon et al. (1998)
744	Furfural	Reverse mutation	S. typhimurium TA100 (modified assay)	426 µg/plate	Negative[a,b]	Kim et al. (1987)
744	Furfural	Reverse mutation	S. typhimurium TA100, TA1535, TA1537 (modified assay)	200 000 µg/ml	Negative	McGregor et al. (1981)
744	Furfural	Reverse mutation	E. coli WP2, WP2 uvrA (modified assay)	0.1–1000 µg/ml	Negative[a,b]	McMahon et al. (1979)
744	Furfural	SOS induction	S. typhimurium TA1535/ pSK1002	1932 µg/ml	Negative[a,b]	Nakamura et al. (1987)
744	Furfural	DNA repair	B. subtilis H17 (rec^+) and M45 (rec^-)	≤ 1000 µg	Negative[a]	Osawa & Namiki (1982)
744	Furfural	DNA repair	B. subtilis H17 (rec^+) and M45 (rec^-)	0.6 ml	Negative[a,b]	Matsui et al. (1989)
744	Furfural	DNA repair	B. subtilis H17	1700–17 000	Positive[a,b]	Shinohara et al.

			(rec$^+$) and M45 (rec$^-$)	µg/disc		(1986)
744	Furfural	Forward mutation	L5178Y mouse lymphoma cells, Tk$^{+/-}$ locus	25–100 µg/ml 200 µg/ml	Negative[a] Positive[a]	McGregor et al. (1988)
744	Furfural	Sister chromatid exchange	Chinese hamster ovary cells	2500–4000 µg/ml	Positive[a,b]	Stich et al. (1981b)
744	Furfural	Sister chromatid exchange	Chinese hamster ovary cells	≤ 1170 µg/ml	Positive[a,b]	National Toxicology Program (1990)
744	Furfural	Sister chromatid exchange	Human lymphocytes	≤ 0.035 mmol/L[a] 0.07– 0.14 mmol/L[c]	Negative[a,b] Positive[a,b]	Gomez-Arroyo & Souza (1985)
744	Furfural	Chromosomal aberration	Chinese hamster ovary cells	500 µg/ml 1000–2000 µg/ml	Negative Positive	Nishi et al. (1989)
744	Furfural	Chromosomal aberration	Chinese hamster ovary cells	≤ 40 mmol/L (3840 mg)	Positive[a,b]	Stich et al. (1981b)
744	Furfural	Chromosomal aberration	Chinese hamster ovary cells	3000 µg/ml	Positive	Stich et al. (1981a)
744	Furfural	Chromosomal aberration	Chinese hamster ovary cells	≤ 1230 µg/ml	Positive[a,b]	National Toxicology Program (1990)
744	Furfural	Unscheduled DNA synthesis	Human liver slices	0.005–10 mmol/L	Negative	Adams et al. (1998)
744	Furfural	Sex-linked recessive lethal mutation	D. melanogaster	1000 mg/kg of diet	Negative	Woodruff et al. (1985)
744	Furfural	Sex-linked recessive lethal mutation	D. melanogaster	100 mg/kg by injection	Positive	Woodruff et al. (1985)
744	Furfural	Sex-linked recessive lethal mutation	D. melanogaster	≤ 6500 mg/kg by injection	Negative	Rodriguez-Arnaiz et al. (1989)
744	Furfural	Chromosomal loss	D. melanogaster	Oral or injected dose of 3750–5000 mg/kg of diet. Mated with repair-proficient females	Negative	Rodriguez-Arnaiz et al. (1992)
744	Furfural	Chromosomal loss	D. melanogaster	Oral or injected dose of 3750–	Positive	Rodriguez-Arnaiz et al.

				5000 mg/kg of diet. Mated with repair-deficient females		(1992)
744	Furfural	Reciprocal trans-location	D. melanogaster	100 mg/kg by injection	Negative	Woodruff et al. (1985)
744	Furfural	Sister chromatid exchange	Mouse bone-marrow cells	50–200 mg/kg bw by injection	Negative	National Toxicology Program (1990)
744	Furfural	Spermhead abnormalities	Mice	4000 mg/kg of diet daily for 5 weeks	Negative	Subramanyam et al. (1989)
744	Furfural	Somatic chromo-somal mutation	Swiss albino mouse bone-marrow cells	1000–2000 mg/kg of diet	Negative	Subramanyam et al. (1989)
				4000 mg/kg bw for 5 days	Positive	
744	Furfural	Sister chromatid exchange	Adult human lymphocytes	9454 mg/m^3 in occupational atmosphere	Negative	Gomez-Arroyo & Souza (1985)
744	Furfural	Chromosomal aberration	Adult human lymphocytes	9454 mg/m^{3}in occupational atmosphere	Negative	Gomez-Arroyo & Souza (1985)
744	Furfural	Unscheduled DNA synthesis	B6C3F1 mice	50–320 mg/kg bw orally	Negative	Edwards (1999)
744	Furfural	Unscheduled DNA synthesis	Fischer 344 rats	5–50 mg/kg bw orally	Negative	Phillips et al. (1997)
745	5-Methylfurfural	Reverse mutation	S. typhimurium TA1537, TA100, TA1535	288 µg/plate	Negative[a,b]	Florin et al. (1980)
745	5-Methylfurfural	Reverse mutation	S. typhimurium TA98, TA100, TA102	96,100 µg/plate	Negative[a,b]	Aeschbacher et al. (1989)
745	5-Methylfurfural	Reverse mutation	S. typhimurium TA98, TA100	79–316 µg/plate	Negative[a,b]	Shinohara et al. (1986)
745	5-Methylfurfural	DNA repair	B. subtilis H17 (rec$^+$) and M45 (rec$^-$)	0.55–5500 µg/disk	Positive[a,b]	Shinohara et al. (1986)
745	5-Methylfurfural	Sister chromatid exchange	Chinese hamster ovary cells	2200–4070 µg/ml	Positive[a,b]	Stich et al. (1981b)

a Without metabolic activation from a 9000 × *g* supernatant of rat liver

b With metabolic activation

c Concentration added to culture

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See Also:

Toxicological Abbreviations

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