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Long-term Inhalation Toxicity of *N*-Vinylpyrrolidone-2 Vapours. Studies in Rats

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Abstract—In previous subchronic studies inhaled *N*-vinylpyrrolidone-2 (NVP) was haemotoxic, hepatotoxic and irritant to the nose. In the first of two long-term studies, study A, Sprague–Dawley rats were exposed by inhalation to 0, 5, 10 or 20 ppm NVP (6 hr/day, 5 days/wk) for 24 months. Satellite groups were killed after 3, 12 or 24 months. In study B, female Sprague–Dawley rats were exposed to 0 or 45 ppm NVP for 3 months and killed at 3 or 12 and 24 months post-exposure. In study A, survival was unaffected, but reduced body weight gain, haemotoxicity, effects on clinical chemistry parameters indicative of hepatotoxicity, increased liver weight, hepatocellular carcinomas, necrosis, reparative hyperplasia, adenomas and adenocarcinomas of the nasal cavity, and squamous cell carcinomas of the larynx were seen. Increased tumour incidence was seen only in the liver and upper respiratory tract. In study B, the effect of NVP on body weight evident at 3 months disappeared before 1 yr, but effects on liver pathology persisted throughout the subsequent 21-month exposure-free period, and a few liver tumours were seen at 2 yr. As NVP gave negative results in a battery of *in vitro* and *in vivo* genotoxicity tests, it appears that the tumours that arose were manifestations of a non-genotoxic mechanism.

Abbreviations: GC = gas chromatography; GSH = glutathione; γ-GT = γ-glutamyltransferase; NVP = N-vinylpyrrolidone-2; THA = total hydrocarbon analyser.

INTRODUCTION

In an accompanying paper (Klimisch *et al.*, 1997), we have described the chemical structure and properties of *N*-vinylpyrrolidone-2 (NVP) (Fig. 1) and referred to its industrial uses and the possibility of workers being exposed to it. In the same paper, we reported findings in 11 subacute/subchronic studies involving repeated exposure of rats, mice or hamsters to NVP by the oral or inhalation routes. These studies revealed that inhaled NVP is irritant to the nasal mucosa of mice and rats and to the tracheal and bronchial epithelium of mice. By contrast, no histopathological evidence of irritancy to the respiratory system was seen in hamsters exposed to 15 ppm for 3 months. Apart from this local toxicity, the main targets for systemic toxicity were the blood and the liver. Decreased haematocrit, decreased haemoglobin and increased platelet counts were seen in several of the studies and effects on liver enzymes, plasma protein levels and the histopathological appearances in the liver were seen in most of the studies in mice and rats. By contrast, increased glycogen was the only effect by exposure to NVP on the livers of hamsters.

NVP gave negative results in a battery of tests for genotoxicity as follows: *in vitro* assays, with or without metabolic activation as appropriate, for point mutations in bacteria (*Salmonella typhimurium* and *Klebsiella pneumoniae*); a test for gene mutations and chromosomal aberrations in mammalian cells (mouse lymphoma assay—L5178Y-HPRT- and TK-loci); a test for unscheduled DNA synthesis and repair in primary cultures of rat hepatocytes; a test for cell transformation in BALB/3T3 cells; a sex-linked recessive lethal assay in *Drosophila melanogaster*; and an *in vivo* micronucleus assay in mice. In addition, *in vivo* studies using differently radiolabelled forms of NVP (^{14}C-labelled either in the C_5-ring position or in the vinyl moiety) indicated no relevant reaction of NVP with DNA (VCI, 1992; Greim, 1994).

In the present paper, two studies are reported. The first of these (study A) was a chronic toxicity/carcinogenicity study in which male and female Sprague–Dawley rats were exposed to 0, 5, 10 or 20 ppm NVP by the inhalation route for 24 months.

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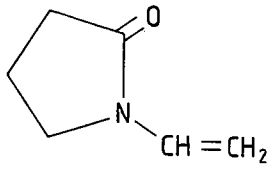

Fig. 1. Chemical structure of *N*-vinylpyrrolidone-2.

The concentrations (dose levels) studied were based on an overview of the findings in previously conducted subacute/subchronic studies (Klimisch *et al.*, 1997). The level of 5 ppm was known from these earlier studies to be irritant to the nasal epithelium. This was deemed to be a local effect, dependent on direct contact between NVP and the affected epithelium. Also, an increased incidence of foci/areas of clear-cell alterations in the liver was seen in response to the inhalation of 5 ppm NVP for 12 months in female, but not male, rats. This was regarded as evidence of toxicity. For these reasons, and since the main aim of the study was to test NVP for possible systemic toxicity and carcinogenicity, 5 ppm was selected as the low-dose level. A 20 ppm concentration was chosen as the high-dose level because the results of the earlier subchronic inhalation studies indicated that this would certainly be toxic but probably consistent with adequate survival to 2 yr. The mid-dose level (10 ppm) was selected because it was the logarithmic mean between 5 and 20 ppm. Study A incorporated subgroups of rats killed after only 3 or 12 months of exposure and also animals exposed for 18 months but not killed until after the elapse of a further 6-month exposure-free period.

The second study (study B) was designed to see whether the liver changes seen in female rats exposed to 45 ppm NVP by inhalation over a 3-month period disappear or progress during a subsequent exposure-free observation period of up to 21 months. This study was completed before it was realized that the nasal cavity and larynx are targets for toxicity by inhaled NVP. Consequently, these tissues were not preserved for histopathological assessment.

MATERIALS AND METHODS

Test chemical

Previous experience established that NVP is stable for 9 months when stored at temperatures of between 5 and 20°C. Every 6 months, a new batch was used for the generation of test atmospheres. The purity of all batches was 99.9%. Before use the compound was stored at a temperature of 15 ± 2°C.

Design of studies

Two long-term studies involving the exposure of Sprague–Dawley rats for 6 hr/day on 5 days/wk were performed. Study A consisted of main groups exposed for 24 months and satellite groups exposed for only 3 months or 12 months, or for 18 months followed by a 6-month exposure-free period (see Table 1). Study B was designed to assess the reversibility of effects seen after exposure for 3 months during subsequent 9-month or 21-month exposure-free periods (see Table 2).

Study A

Animals, maintenance, and the inhalation exposure system. Specified pathogen-free male and female Sprague–Dawley rats (CRL:CD (SD) Br, 37–39-days-old) were purchased from Wiga, Charles River (Sulzfeld, Germany). They were identified individually by an ear-tattoo number, housed two per cage in stainless-steel wire cages and maintained in identical horizontal-flow stainless-steel inhalation chambers (Fig. 2, V = 4.2 m^3). The chambers were composed in a modular construction: each chamber consisting of three modular compartments, with 36 cages. Each module can be used as individual inhalation chamber after appropriate installation with additional flow tunnels. Such an inhalation system is very flexible and adaptable to different study types (one module for up to 72 rats for subchronic studies; a three-module chamber for up to 216 rats for carcinogenicity studies). Each inhalation chamber was installed in a separate air-conditioned room maintained at 22 ± 2°C with a relative humidity of 55 ± 10% and a 12-hr light/dark cycle (light between 06.00 and 18.00 hr). At weekends, the animals were removed from the chambers and transferred to racks in the same room. Maintenance diet (10-mm diameter pellets, Klingentalmühle AG, Germany) and drinking water were provided *ad lib.* between exposure periods, but withdrawn from the animals during exposure. Each batch of diet was assayed for bacterial and chemical contaminants. The drinking water was similarly assayed at regular intervals. The results of these analyses provided no

Table 1. Test groups: study A

NVP target concn (ppm)	Main group no. of animals		Satellite group 1[1] no. of animals		Satellite group 2[2] no. of animals		Satellite group 3[3] no. of animals	
	Females	Males	Females	Males	Females	Males	Females	Males
0	70	70	10	10	10	10	10	10
5	60	60	20	20	10	10	10	10
10	60	60	20	20	10	10	10	10
20	60	60	20	20	10	10	10	10

[1]Interim kill after 3-month exposure.
[2]Interim kill after 12-month exposure.
[3]Exposure for 18 months followed by a 6-month post-exposure observation period.

Table 2. Test groups, NVP concentration of study B

Test group	NVP target concn (ppm)	NVP analytical concn ± SD (ppm)	No. of animals (main group)[1]	No. of animals (Satellite group 1)[2]	No. of animals (Satellite group 2)[3]
1	—	—	15	10	10
2	45	45.4 ± 0.84	15	10	10

[1]Main group = 3-month exposure followed by 21-month post-exposure observation period.
[2]Satellite group 1 = 3-month exposure.
[3]Satellite group 2 = 3-month exposure followed by a 9-month post-exposure observation period.

evidence of contamination that might have jeopardized the quality of the study.

Test groups and target concentrations. The animals were assigned randomly to the test groups (Table 1) using a computer-based randomization plan. The main group was exposed for 24 months and the three satellite groups for 3 months (satellite group 1), 12 months (satellite group 2) and 18 months (satellite group 3), respectively. NVP concentrations (Table 1) were based on the results of previously completed subchronic toxicity studies (see above and Klimisch *et al.*, 1997). The choice of 5 ppm for the low concentration group was based on the expectation on the findings in the earlier studies that this was likely to be on the borderline of being a no-effect-level for histopathological changes in the liver, although experience indicated that evidence of irritation to the nasal mucosa was still to be expected in response to this level of exposure.

Generation and analysis of NVP inhalation atmospheres. In order to facilitate the evaporation of NVP under mild conditions with respect to temperature (45–55°C), an ultra-fine NVP aerosol was generated using a two-component atomizer and compressed air (Fig. 3). The atomizer was connected to an all-glass evaporator and heated by a thermostat-controlled water-bath. The generated vapours were led through a glass frit D2 to ensure that no aerosol could reach the inhalation chambers. The absence of aerosol particles was controlled by the use of a particle analyser (Malvern API aerosizer). The vapours were diluted with conditioned air to the desired concentrations and delivered to the chambers at a flow rate of 80–90 m^3/hr. The control chamber was operated under slight positive pressure and the NVP group under slight negative pressure by pressure-controlled adjustment of an exhaust air system. All flow rates, pressure conditions, relative humidity and temperatures were measured continuously and regulated by an automatically-operated computerized control and regulation system (for details see Table 3). The values were monitored on a TV screen, scanned about every 12 sec, changed from analogue to digital and fed to a PC system. The PC system compared the incoming data with preset limit values and gave an alarm when these values were overrun. Hourly means and daily means with standard deviations were printed out in daily records. In Table 3, the overall study means of different parameters and the analytically measured concentrations are given.

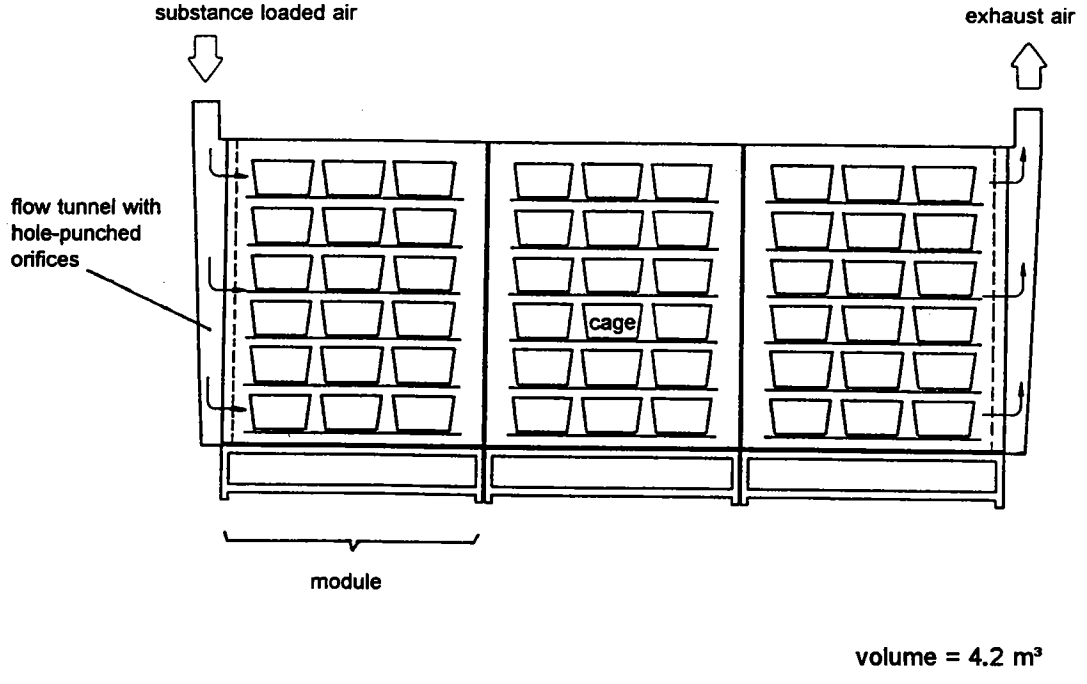

Fig. 2. Inhalation chamber (horizontal flow).

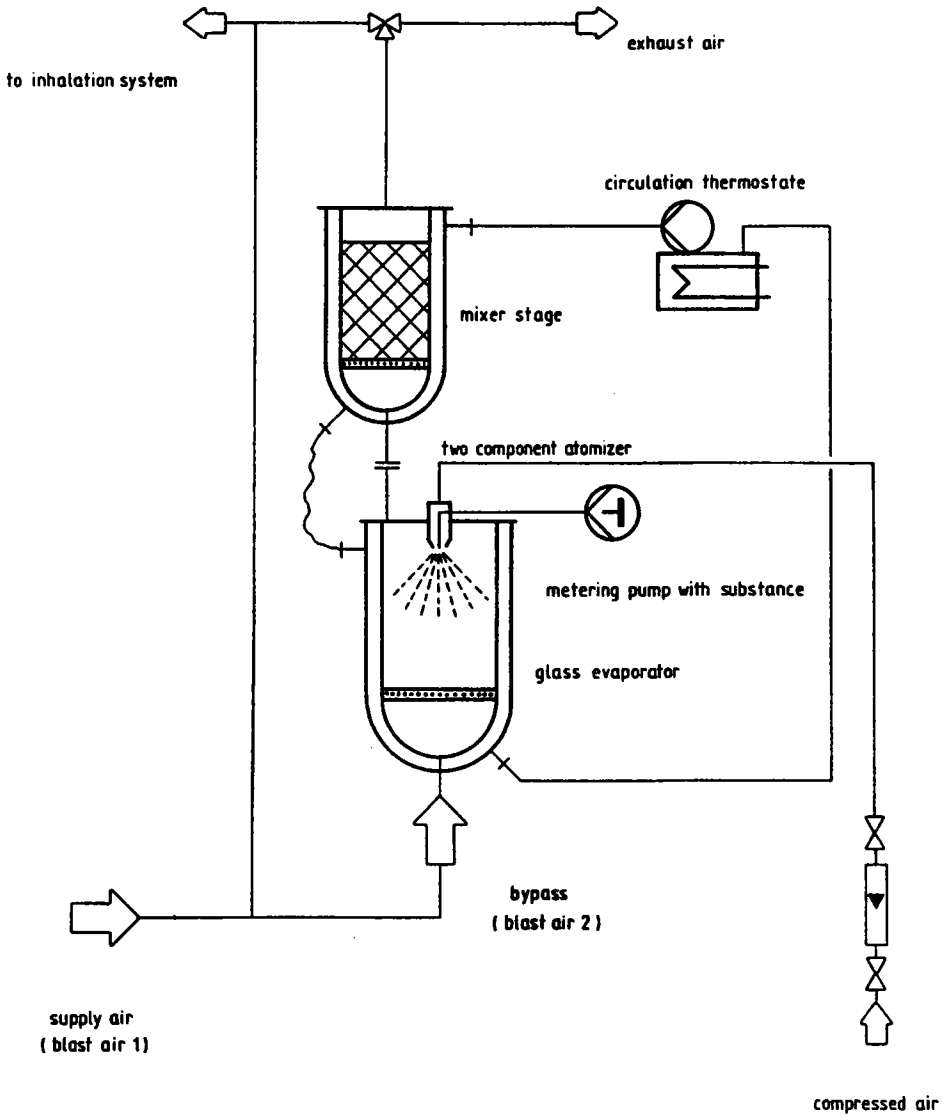

Fig. 3. The generation of NVP vapour.

The NVP concentrations in the inhalation chambers were monitored using a total hydrocarbon analyser (THA; Ratfisch RS 55, München, Germany). The THA was calibrated using NVP–air mixtures of analytically measured concentrations. Samples of NVP–air mixtures were absorbed in two fritted-glass flasks filled with 15 ml *n*-propanol and analysed with a gas chromatograph (GC) (1 m glass column, 2 mm diameter) with 15% Carbowax 20 M +3% KOH on Chromosorb WAW DMCS/HP, 80/100 mesh; Hewlett Packard GC 5840 A with automatic sampler; FID temperature 250°C, oven temperature 135°C, carrier gas flow rate 29.8 ml/min; calibrated retention times: NVP = 4.9 min; internal standard (C_{17}–paraffin = 4 min). The performance of THA was checked weekly using a test gas (propane in nitrogen). The GC was calibrated using weighed amounts of liquid NVP. Every month, the NVP chamber atmospheres were analysed additionally using a GC procedure to verify the THA concentrations. There was close concordance between the results based on the two methods of measure-

Table 3. NVP concentrations and chamber parameters: study A

NVP target concn (ppm)	Analytically measured concn ± SD (ppm NVP)	Range of relative humidity (%)	Chamber temperature range (°C)	Chamber pressure (Pa)
0	—	43–65	21–25	2–9
5	5.0 ± 0.10	43–64	21–25	(–2)–(–7)
10	10.0 ± 0.14	40–62	21–25	(–2)–(–8)
20	20.2 ± 0.35	36–59	20–25	(–2)–(–10)

ment and no compounds other than NVP were detected by the GC in the three inhalation chambers. In the control chamber no NVP was found (detection limit, 0.01 ppm).

Animal observation, haematological, clinical chemistry and histological assessment. Animals were weighed once weekly during the first 3 months and monthly thereafter. During exposure, animals were carefully observed through glass windows of the chambers. Before and after the 6-hr exposure period on each exposure day and twice on non-exposure days, a check was made for moribund or dead animals. Additionally, each animal was gently palpated and visually examined once every 4 wk. The following examinations were carried out:

1. Satellite group 1 (3-month exposure) and satellite group 2 (12-month exposure)

Ophthalmologic examination for changes to the refracting media using a focusable Heine–Focalux hand-held slit lamp before the first exposure and shortly before terminal kill.

Blood was randomly taken by retro-orbital venipuncture for haematological and clinical chemistry parameters from non-fasted animals before they were killed. Haematological and clinical chemistry parameters were closely similar to those listed for the subchronic studies (see Klimisch *et al.*, 1997). In liver, homogenates γ-glutamyltransferase (γ-GT) activities and glutathione (GSH) concentrations were determined.

Animals were killed by exsanguination from the abdominal aorta and vena cava under Narcoren® anaesthesia. They were necropsied and assessed for gross pathology. Liver, kidneys, adrenal glands, lungs, brain and testes were weighed. The list of organs and/or tissues fixed in 4% formaldehyde solution was similar to that in the subchronic studies (Klimisch *et al.*, 1997) with the addition of the head with nose, nasal cavity and pharynx. In the case of the liver, samples were also fixed in: (a) Carnoy's solution; and (b) an acetic acid–ethanol solution.

Liver, nasal cavity (four levels), pancreas and gross lesions were embedded in paraffin and stained with haematoxylin and eosin. Further sections of the liver were stained for glycogen, γ-GT and fat. Microscopic examination was conducted on these tissues from all animals.

The four levels of sectioning of the nasal cavity in the present study were as follows: I—just posterior to the upper incisor teeth; IV—at the level of the second molar teeth and showing ethmoid turbinates; levels II and III—intermediate between I and IV and including areas covered by respiratory as well as olfactory epithelium.

2. Satellite group 3 (18 month exposure followed by 6-month post-exposure observation period)

Ophthalmoscopy, liver biochemistry, GSH concentrations and γ-GT activity in liver homogenate, selected organ weights, gross pathology, and histopathology (details as for satellite groups 1 and 2). In addition, urine samples were taken for routine urinalyses.

3. Main groups (24-month exposure)

Blood smears were prepared from all surviving animals at the end of the study and from animals killed in a moribund state.

The following examinations were carried out: differential white blood cell count (all animals killed); organ weight determinations, gross pathology (details as for satellite group 1). Liver, nasal cavity, larynx, trachea, lungs, pancreas, kidneys and gross lesions were examined histopathologically for all animals. In addition, all other fixed tissues were examined for animals that died or were killed moribund during the study and for all terminally-killed animals in the control and 20 ppm groups.

Statistical analyses. For body weight, haematology, clinical chemistry parameters and organ weights, analysis of covariance was carried out using ANOVA. If a significant difference was observed between groups the mean values were compared by Dunnett's test. Where there were less than eight animals per group, a statistical one-sided analysis was carried out using the Kruskal–Wallis *t*-test. The incidences of lesions were analysed according to the PETO analysis (ROELEE-84 System).

Study B

Animals. Specific pathogen-free female Sprague–Dawley rats (CAW/ICO/WIGA; 7 wk old; Charles-River-Wiga GmbH, Sulzfeld, Germany), allocated by a computer-based randomization method to the test groups as shown in Table 2.

Maintenance, exposure conditions and clinical monitoring of animals. These were essentially the same as those described for study A.

Observations. Animals were individually weighed once weekly during the first 3 months and thereafter monthly. Serum levels of GSH and γ-GT were determined in blood samples taken immediately prior to sacrifice. γ-GT was also measured in liver homogenates. Pieces from three different liver lobes were fixed in Carnoy's solution, embedded in paraffin wax and stained with haematoxylin and eosin and for glycogen. The remaining liver was fixed in 4% formaldehyde solution and one section was stained for fat. All liver slides were examined by light microscopy. No other organs/tissues were examined histologically in study B.

RESULTS

Study A

This report focuses on the findings in the main groups of animals. Where treatment-related effects were seen in main group animals, relevant findings in satellite groups are briefly summarized in the present paper. Detailed data derived from satellite animals have been presented in the paper describing

the effects of subchronic exposure to NVP (Klimisch *et al.*, 1997).

Analysis of concentrations of NVP in chambers

The concentrations found in the 5 ppm chamber ranged from 4.86 to 5.20 ppm with a mean of 5.0 ppm ± 0.01 SD. Those in the 10 ppm chamber ranged from 9.80 to 10.37 ppm with a mean of 10.0 ppm ± 0.14 SD. Those in the 20 ppm chamber ranged from 19.66 to 20.72 ppm with a mean of 20.1 ppm ± 0.30 SD.

Body weight (main group animals)

Figure 4 (A, B) illustrates the effects of exposure to different concentrations of NVP on body weight. Significantly reduced body weight gain was observed in response to all three concentration levels of NVP. In response to 5 ppm NVP the reduction in males was significant from wk 2 and after 3 months the decrease was 5.9% compared with the controls. In females exposed to 5 ppm, the reduction was significant from wk 3 and after 3 months the decrease was 3.4% compared with controls. In response to 10 ppm, significant reductions were evident after 1 and 2 wk, respectively, in males and females and at 3 months the decreases were 6.5% in males and 5.6% in females. In response to 20 ppm, significant reduction in body weight was evident in both sexes after 1 wk, and at 3 months the decreases amounted to 11% in males and 5% in females.

Later in the study, the differences between the treated and control groups remained small or non-existent.

Food consumption (satellite groups)

No treatment-related effects were observed.

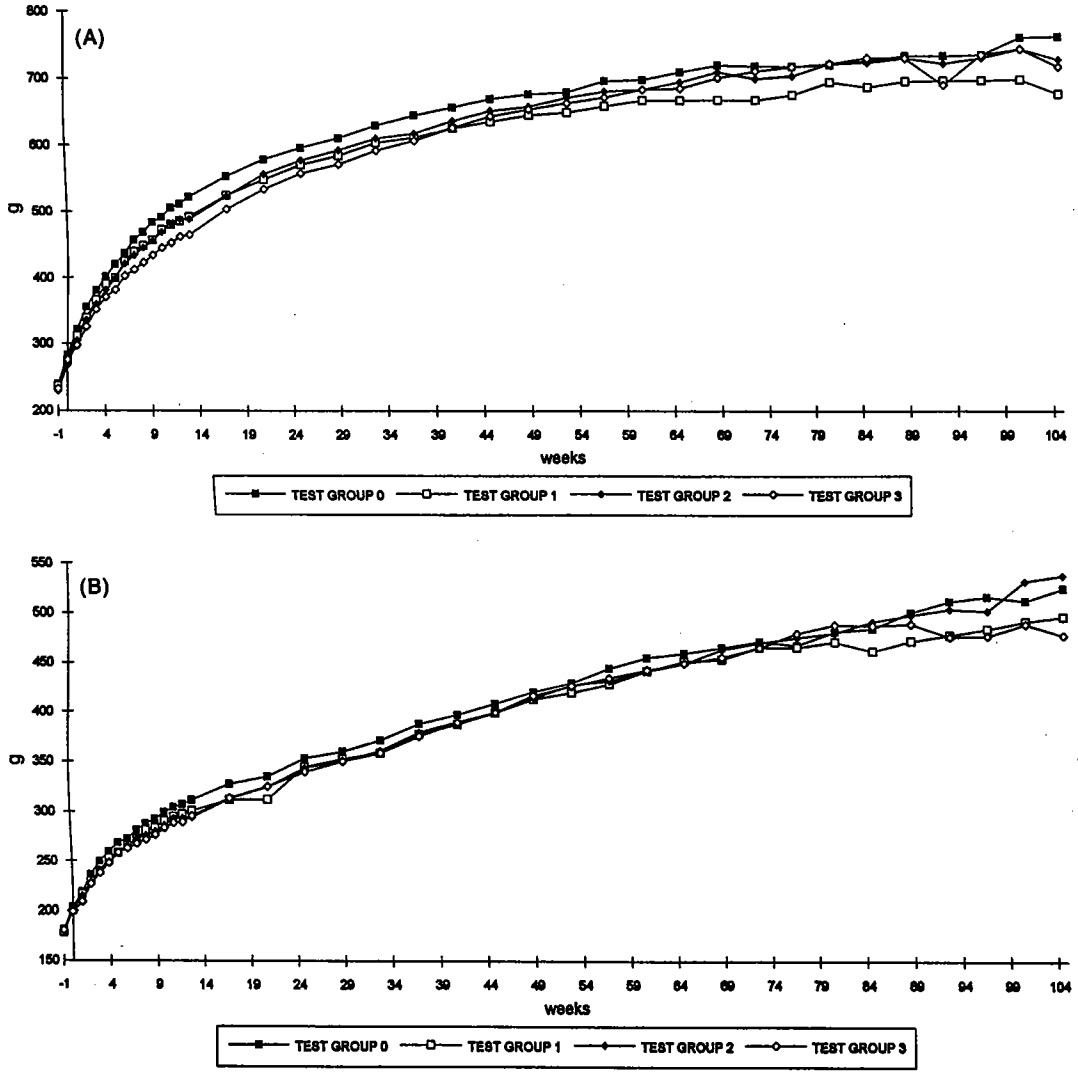

Fig. 4. **Study A**: effect of exposure to NVP on body weight in (A) male and (B) female rats (statistically significant differences are given in the text).

Table 4. Cumulative incidences of subcutaneous and generalized abdominal swellings in main group rats in study A

Sex	Males				Females			
Group	0	1	2	3	0	1	2	3
NVP (ppm)	0	5	10	20	0	5	10	20
No. of rats	70	60	60	60	70	60	60	60
Subcutanous swelling	12	10	14	17	37	36	33	27
Abdominal swelling	2	4	1	3	2	1	1	2

Clinical observations (main groups)

In neither sex was the incidence of any clinically evident change significantly higher in any treated group than in the control group. The cumulative incidences of palpable subcutaneous swellings and abdominal swellings were as shown in Table 4.

Survival (main group)

Throughout the study exposure to NVP at any dose level was without statistically significant effect on survival in either sex (see Fig. 5A and B).

Haematological findings (main groups)

In main-group animals killed at the termination of the study at 2 yr, increased incidences of polychromasia, anisocytosis, microcytosis and macrocytosis of erythrocytes were seen in females, particularly in those exposed to 10 or 20 ppm (Table 5). No statistically significant treatment-related effects were seen on red blood cell parameters in males and none were seen on white blood cell parameters in either sex. A similar pattern of findings was seen in Satellite 2 animals killed at 1 yr (Table 5).

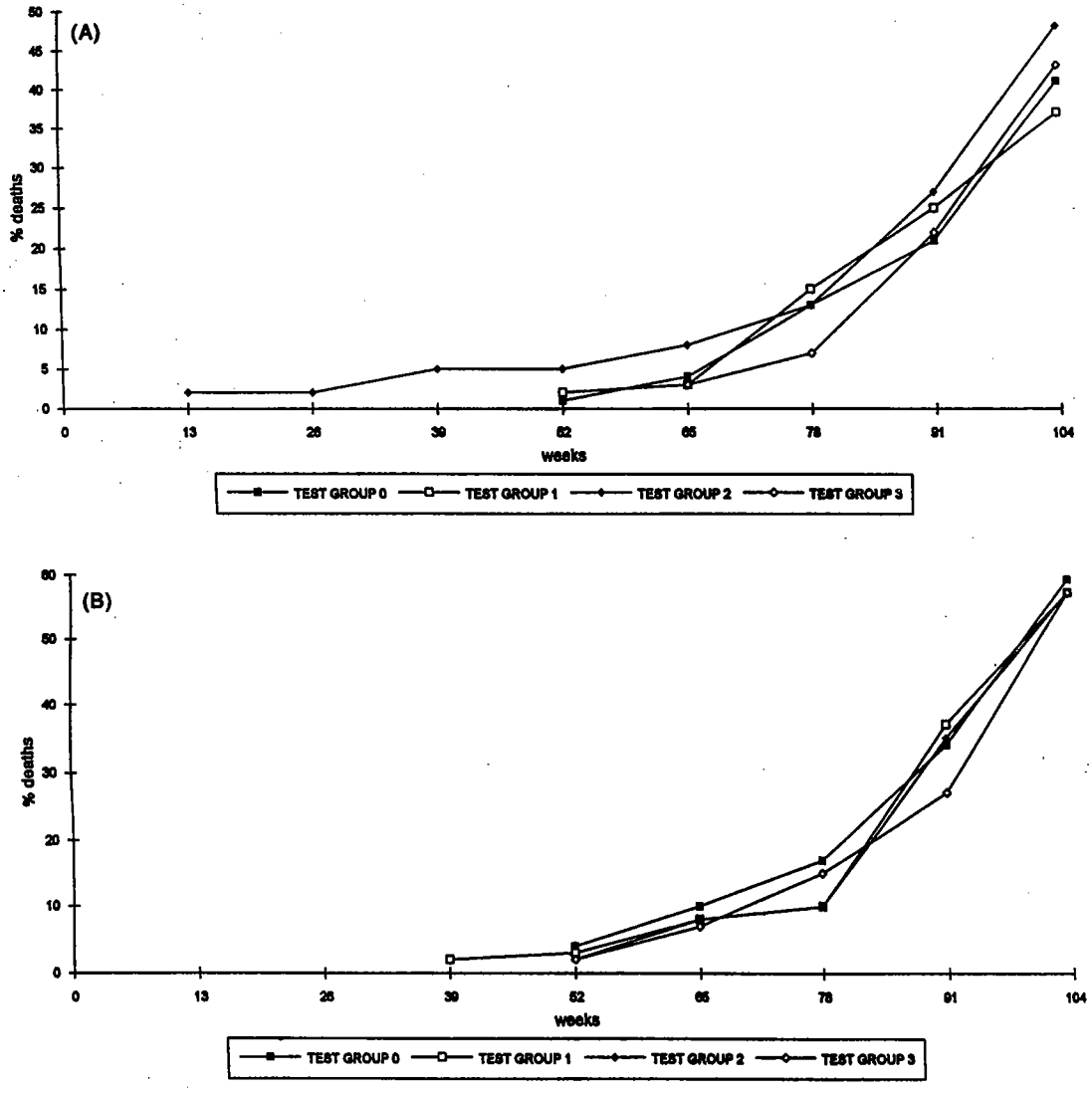

Fig. 5. **Study A**: cumulative lethality in (A) male and (B) female rats.

Table 5. Percentage incidence of morphological changes in red blood cells in main group rats at 2 yr and in Satellite group 2 animals killed at 1 yr (study A)

Sex	Males				Females				Trend
Group	0	1	2	3	0	1	2	3	
ppm NVP	0	5	10	20	0	5	10	20	
Satellite 2 animals killed at 1 yr									
No. of animals	10	10	10	10	10	10	10		
Polychromasia	0	0	10	0	0	0	0	10	
Microcytosis	0	0	0	10	10	0	10	50	+
Anisocytosis	0	0	20	30	10	20	10	60	+
Main groups killed at 2 yr									
No. of survivors at 2 yr	39	38	30	34	29	25	26	25	
Polychromasia	5.13	5.25	6.67	8.82	0	8.00+	11.54++	8.00+	++
Microcytosis	0	0	0	2.94	6.90	16.00+	46.15++	52.00++	+++
Macrocytosis	2.56	2.63	6.67	8.82	6.90	4.00	15.38+	20.00++	+++
Anisocytosis	—	—	—	—	6.90	12.00	23.08	40.00	+++

+ = $P < 0.05$; ++ = $P < 0.01$; +++ = $P < 0.001$.

Certain other haematological changes were seen in satellite groups 1 and 2 earlier in the study. These included (i) an increase in platelet count at 3 months in animals of both sexes exposed to 10 ppm or 20 ppm NVP; (ii) increased mean corpuscular haemoglobin concentration, reticulocytes, total white cell counts, lymphocytes, neutrophils and monocytes at 12 months in females exposed to 20 ppm; and (iii) decreased haemoglobin, haematocrit, and mean corpuscular volume (MCV) at 12 months in females exposed to 20 ppm NVP.

Clinical chemistry (satellite 1 and 2 animals)

The clinical chemistry findings at 3 months (satellite groups) and at 12 months (satellite groups) are summarized in Table 6.

Increases in hepatic GSH were seen at 3 months in rats of both sexes exposed to 20 ppm NVP and in males exposed to 10 ppm NVP. Females exposed to 20 ppm also showed γ-GT at this time. These effects on liver chemistry at 3 months were accompanied by reduced plasma levels of total protein and globulin in rats of both sexes and decreased albumin concentration in females at all three dose levels. Most of these effects are indicative of hepatotoxicity in both sexes, especially in females. By contrast, the treatment-related reductions in alanine transferase activities at 3 months and 12 months (Table 6) are unexpected in response to a hepatoxin.

Similar effects were seen in satellite 2 rats killed after 12 months' exposure to NVP. However, they were somewhat less marked than at 3 months. In addition, at 12 months, females exposed to 20 ppm NVP had a significantly raised blood cholesterol level.

Urinalysis

Urine samples from satellite group 3 animals were taken for analysis after 3, 6 and 12 months. No effects of exposure to NVP were seen in any of these analyses.

Organ weights

The only meaningful treatment-related differences in organ weights at 2 yr were statistically significant higher ($P < 0.01$) mean absolute and relative liver weights in both sexes in response to 20 ppm NVP (Table 7). A significantly lower mean absolute brain weight in males exposed to 20 ppm was not ac-

Table 6. Clinical chemistry findings: significant differences from controls at 3 and 12 months (study A)

Sex	Males			Females		
Group	1	2	3	1	2	3
NVP (ppm)	5	10	20	5	10	20
At 3 months (satellite group 1)						
ALT				—	—	−
LGGT						++
GSH		++	++			++
Total protein	−		—	—	−	—
Albumin				—	—	—
Globulin	−	−	—	—		—
At 12 months (satellite group 2)						
ALT					−	−
LGGT						++
GSH	++		+			++
Total protein					—	
Albumin			++		—	—
Globulin					—	
Cholesterol						+

ALT = alanine aminotransferase; LGGT = liver γ-glutamyltransferase; GSH = reduced glutathione in the liver;
+ or − = $P < 0.05$ higher or lower than in controls; ++ or −− = $P < 0.01$ higher or lower than in controls.
Blank spaces denote no significant difference between exposed and control groups.

Table 7. Effect of exposure to NVP on absolute and relative liver weights in main group animals

	Absolute (g)				Relative to body weight (%)			
Group	0	1	2	3	0	1	2	3
NVP (ppm)	0	5	10	20	0	5	10	20
				Males				
No. of rats	39	36	29	33	39	36	29	33
Mean body weight	740	662–	710	696				
Mean liver weight	22.1	19.1	23.6	31.0++	2.9	2.9	3.3	4.5++
Mean brain weight	2.4	2.4	2.4	2.3—	0.33	0.36	0.34	0.34
				Females				
No. of rats	29	25	26	24	29	25	26	29
Mean body weight	503	477	522	463				
Mean liver weight	16.3	16.1	18.9	24.0++	3.2	3.4	3.7	5.3++
Mean brain weight	2.2	2.2	2.2	2.1	0.46	0.47	0.43	0.47

– or + = $P < 0.05$; — or ++ = $P < 0.01$.

companied by any between-group differences in brain weight relative to body weight.

The effects of exposure to NVP on liver weight in main group animals were matched by (i) statistically significant and dose-dependent increases in mean absolute liver weight in satellite group 1 males killed after 3 months' exposure to 10 or 20 ppm NVP; (ii) statistically significantly higher mean absolute and relative liver weights in females killed after 3 months' exposure to 20 ppm NVP; (iii) statistically higher mean absolute liver weights in males exposed to 5, 10 or 20 ppm NVP for 1 yr; (iv) statistically higher mean relative liver weight in satellite group 2 males exposed to 20 ppm for 1 yr; (v) statistically significant and dose-dependent higher mean absolute liver weight in females exposed to 10 or 20 ppm NVP for 1 yr; and (vi) significantly higher mean relative liver weight in females exposed to 20 ppm NVP for 1 yr. In satellite group 3 animals exposed to NVP at the three different dose levels for 18 months and thereafter kept under observation, no between-group differences in absolute or relative liver weights were seen.

Microscopic findings

The protocols for the study required that a full range of tissues would be examined microscopically in the case of (i) all main-group high-dose and control animals; (ii) all decedents exposed to 10 ppm or 5 ppm NVP; (iii) all tissues from terminally-killed mid-dose and low-dose animals in which an adverse treatment-related effect was suspected on the basis of macroscopic and/or microscopic observations of animals in groups 0 and 3; and (iv) tissues in terminally-killed mid-dose and low-dose animals considered to be abnormal at necropsy. Compliance with this protocol resulted in the following tissues being examined microscopically in all animals: kidney, larynx, liver, lung, nasal cavity, pancreas and trachea. However, it transpired that only three of these tissues—larynx, liver and nasal cavity—were, in fact, targets for toxicity.

Table 8(A, B) lists the findings in respect of neoplasms in all animals in the study. The lower incidence of pituitary adenomas in the males of low-dose and mid-dose animals is consistent with the possibility that small tumours in terminally-killed animals that were not detected macroscopically were not sectioned.

In the cases of the larynx, liver and nasal cavity, exposure to NVP clearly exhibited treatment- and dose-related effects on tumour incidence. As shown in Table 8B, for sites other than the nose, larynx and liver, exposure to 20 ppm NVP had no adverse effect on the incidence of benign or malignant neoplasms at one or more sites. The statistical significance of the between-group differences is shown in Table 9 and is further discussed below.

The pattern of incidence of non-neoplastic lesions in tissues not regarded as targets for NVP toxicity showed no evidence of any effect of exposure. Table 10 summarizes the findings in respect of the tissues of which samples were examined microscopically in all animals at all dose levels.

In the kidney, exposure to NVP was associated with dose-related reductions in the incidences of nephropathy and tubular hyperplasia. In the larynx, a few mid-dose and high-dose animals exhibited epithelial hyperplasia. In the liver, exposure to NVP was associated with increased incidences of focal hyperplasia, focal cellular alteration, centrilobular fatty infiltration (males only) and decreased lymphoid infiltration (males only). The most striking hepatic effects were on the incidences of focal eosinophilic hyperplasia in females and eosinophilic foci of hepatocellular alteration in both sexes. Spongiosis hepatis was seen in higher incidence in mid-dose and high-dose animals of both sexes and in low-dose females. In the lungs the only significant between-group difference seen was a lower incidence of foam cells in high-dose females. In the nasal cavity, inflammation, atrophy of olfactory epithelium, basal cell hyperplasia, hyperplasia of glands, goblet cell hyperplasia, hyperplasia of olfactory epithelium and epithelial metaplasia were all seen in higher incidence in NVP-exposed animals than in controls and in most cases these effects were dose related.

The data shown in Tables 9 and 10 combine the findings at all four levels of sectioning of the nasal cavity (see Materials and Methods). However, such combination of data obscures the fact that the findings varied in different parts of the nasal cavity and in relation to the types of epithelium with which

Table 8A. Incidence of primary neoplasms in main group animals (decedents and terminally-killed animals combined) in study A

Sex			Males				Females			
Group			0	1	2	3	0	1	2	3
NVP (ppm)			0	5	10	20	0	5	10	20
No. of animals in group			70	60	60	60	70	60	60	60
Organ	Type of neoplasm	B or M	Males				Females			
Tissues examined microscopically in all main group animals										
Kidney	Transitional cell carcinoma	M	0	0	0	0	1	0	0	0
	Transitional cell papilloma	B	1	0	0	0	0	0	0	0
	Mesenchymal tumour	M	0	0	1	0	0	0	0	0
	Lipomatous tumour	B	0	0	1	0	0	0	0	1
		M	0	0	0	0	0	0	1	0
Larynx	Squamous carcinoma	M	0	0	0	4	0	0	0	4
Liver	Hepatocellular carcinoma	M	1	6	5	17	1	3	6	26
	Haemangiosarcoma	M	0	0	0	0	0	0	0	3
	Cystadenoma, biliary	B	0	0	0	1	0	0	0	2
	Leiomyoma	B	1	0	0	0	0	0	0	0
Lung	Squamous carcinoma	M	0	0	1	0	0	0	0	0
	Adenoma	B	1	1	0	4	0	0	2	0
Nasal cavity	Adenocarcinoma	M	0	0	4	6	0	0	0	4
	Adenoma	B	0	8	9	10	0	2	8	12
	Haemangiopericytoma	B	0	0	1	0	0	0	0	0
Pancreas	Islet-cell carcinoma	M	0	2	1	1	0	1	2	1
	Islet-cell adenoma	B	9	10	11	11	6	4	3	7
	Exocrine adenoma	B	0	0	1	1	0	0	0	0
	Mixed tumour	B	1	0	4	0	0	0	0	0
Tissues examined in all animals except terminally-killed groups 1 and 2 if apparently normal macroscopically										
Adipose tissue	Liposarcoma	M	0	0	0	1	0	0	0	0
	Lipoma	B	3	3	3	3	0	1	0	0
Adrenal cortex	Adenoma	B	0	2	0	0	0	2	2	0
Adrenal medulla	Tumour	B	6	6	2	6	5	2	3	3
		M	0	1	0	1	0	0	0	0
	Ganglioneuroma	B	1	0	0	0	0	0	0	0
Bone	Osteosarcoma	M	0	1	0	1	0	0	0	0
Brain	Glioma	B	1	0	0	0	0	0	0	0
		M	1	1	0	0	1	0	0	0
	Meningioma	B	0	1	0	0	0	0	0	0
Haemopoietic	Lymphoma	M	5	1	4	3	1	0	1	0
	Histiocytoma, fibrous	M	2	1	2	2	0	2	1	2
	Histiocytic sarcoma	M	1	0	1	0	0	0	0	0
	Granulocytic leukaemia	M	0	0	0	0	0	0	0	1
Lacrimal gland	Adenocarcinoma	M	1	0	0	0	0	0	0	0
	Adenoma	B	0	0	0	0	0	1	0	0
Mammary gland	Adenocarcinoma	M	0	0	0	0	10	13	14	9
	Carcinoma in fibroadenoma	M	0	0	0	0	0	4	6	1
	Fibrosarcoma	M	0	1	1	0	1	1	1	0
	Mixed tumour (malignant)	M	0	0	0	0	1	0	0	0
	Adenoma	B	0	0	0	0	5	3	2	4
	Fibroadenoma	B	0	0	0	0	28	22	24	19
	Fibroma	B	1	0	1	2	6	8	2	1
	Pericytoma	M	0	0	0	0	0	0	1	0
Mesenteric lymph node	Haemangioma	B	0	0	0	0	0	0	0	1
Ovary	Sertoli cell	M	—	—	—	—	0	0	1	0
	Thecoma	B	—	—	—	—	0	0	1	0
Parathyroid	Adenocarcinoma	M	0	0	0	1	0	0	0	1
	Adenoma	B	1	0	0	2	0	0	0	0
Pituitary	Adenocarcinoma	M	2	0	0	0	5	2	2	3
	Adenoma	B	46	27	32	43	55	47	51	44
Preputial gland	Squamous carcinoma	M	0	0	0	1	0	0	0	0
Skin and subcutaneous	Squamous carcinoma	M	1	1	0	2	2	0	1	1
	Basal cell tumour	B	1	0	0	0	0	0	0	0
	Keratoacanthoma	B	1	3	1	1	0	0	0	0
	Squamous papilloma	B	0	0	1	1	0	0	0	0
	Sebacous adenoma	B	1	1	0	0	0	0	0	0
	Hair follicle tumour	B	1	0	1	0	0	0	0	0
	Fibrosarcoma/sarcoma	M	1	0	1	2	0	0	1	1
	Schwannoma	B	0	0	1	0	0	0	0	0
Spleen	Haemangiosarcoma	M	0	0	0	0	0	0	0	0
Stomach—fore	Squamous carcinoma	M	0	1	1	0	0	0	0	0
	Schwannoma	M	0	0	0	1	0	0	0	0
Stomach—glandular	APUD cell	B	0	0	0	1	0	0	0	0
Testis	Leydig cell	B	3	3	2	1	—	—	—	—
Thymus	Liposarcoma	M	0	1	0	0	0	0	0	0
	Thymoma	M	0	0	0	0	1	0	0	0
Thyroid	Adenocarcinoma	M	0	3	0	1	0	0	0	0
	Adenoma	B	1	0	0	1	1	0	0	0
	C-cell tumour	B	4	1	2	3	1	1	0	3
		M	0	0	2	0	1	1	0	0
Tongue	Basal-cell tumour	B	0	0	0	1	0	0	0	0
Uterus	Squamous carcinoma	M	—	—	—	—	0	1	1	2
	Carcinosarcoma	M	—	—	—	—	0	1	0	0
	Leiomyosarcoma	M	—	—	—	—	1	0	0	0
	Schwannoma	M	—	—	—	—	1	1	0	1

B = benign M = malignant

Table 8B. Numbers and percentages of main group animals that developed neoplasms at one or more sites in study A

No. (%) of animals with:	Group (concentration NVP)							
	0	1 (5 ppm)	2 (10 ppm)	3 (20 ppm)	0	1 (5 ppm)	2 (10 ppm)	3 (20 ppm)
	Males				**Females**			
One or more benign or malignant neoplasms	64 (91.4)	—	—	59 (98.3)	69 (98.6)	—	—	58 (96.7)
Two or more benign of malignant neoplasms	27 (38.6)	—	—	42 (71.2)	39 (55.7)	—	—	52 (86.7)
One or more malignant neoplasms	14 (20.0)	—	—	33 (55.0)	25 (35.7	—	—	42 (70.0)
One or more benign or malignant neoplasms other than of larynx, liver (hepatocullular) or nasal cavity	64 (91.4)	—	—	57 (95.0)	69 (?)	—	—	58 (?)
One or more malignant neoplasms other than of the larynx, liver (hepatocellular) or nasal cavity	13 (18.6)	—	—	17 (28.3)	25 (35.7)	—	—	22 (36.7)
Total no. of tumours:								
Benign or malignant	105	—	—	139	134	—	—	161
Benign	88	—	—	94	107	—	—	99
Malignant	17	—	—	45	27	—	—	62
Total no. of tumours other than of the larynx, liver (hepatocellular) or nasal cavity:								
Benign or malignant	104	—	—	102	133	—	—	115
Benign	88	—	—	84	107	—	—	87
Malignant	16	—	—	18	26	—	—	28
Average no. of tumours per animal:								
Benign or malignant	1.5	—	—	2.3	1.9	—	—	2.7
Benign	1.3	—	—	1.6	1.5	—	—	1.6
Malignant	0.24	—	—	0.75	0.39	—	—	1.03
Average no. of tumours other than of the larynx, liver (hepatocellular) or nasal cavity:								
Benign or malignant	1.5	—	—	1.7	1.9	—	—	1.9
Benign	1.3	—	—	1.4	1.5	—	—	1.5
malignant	0.23	—	—	0.30	0.37	—	—	0.47

Table 9. Observed vs expected incidences of nasal cavity and liver neoplasms in main groups, Satellite group 1 and 2 animals and Satellite group 3 animals that died before 18 months in study A

		Group (concentration NVP)				Total	Trend
		0	1 (5 ppm)	2 (10 ppm)	3 (20 ppm)		
Nasal cavity							
Adenoma				**Males**			
	O	0	9	9	11	29	
	E		4.13	4.00	5.3		
	p		++	+++	+++	**	++
				Females			
	O	0	2	8	14	24	
	E		0.93	3.88	6.92		
	p		NS	++	+++	***	+++
Adenocarcinoma				**Males**			
	O	0	0	4	6	10	
	E			1.91	2.93		
	p		NS	+	+	*	+++
				Females			
	O	0	0	0	4	4	
	E				2.08		
	P		NS	NS	(+)	*	++
Liver							
Hepatocelluar carcinoma				**Males**			
	O	1	6	5	17	29	
	E		3.36	2.56	8.64		
	p		+	+	+++	***	+++
				Females			
	0	1	3	6	26	36	
	E		1.68	3.12	12.40		
	p		NS	+	+++	***	+++

O = observed; E = expected in direct comparison with control group.
+ = $P<0.05$; ++ = $P<0.01$; +++ = $P<0.001$.

Table 10. Incidence of selected non-neoplastic lesions in main group animals (decedents and terminally-killed animals combined) in study A*

Sex			Males				Females			
Group			0	1	2	3	0	1	2	3
NVP (ppm)			0	5	10	20	0	5	10	20
No. of animals in group			70	60	60	60	70	60	60	60
Organ	Type of neoplasm		Males				Females			
	Tissues examined microscopically in all animals									
Kidney	Nephropathy		48	46	35	40	45	35	33	24
	Tubular hyperplasia		14	9	6	8	7	3	4	1
Larynx	Epithelial hyperplasia		0	0	3	6	0	0	0	4
	Inflammation		6	4	4	4	0	2	2	5
Liver	Focal hyperplasia	Any	3	8	14	21	5	15	20	28
		Basophil	3	6	9	14	5	14	13	9
		Clear cell	0	1	1	4	0	0	1	1
		Eosinophil	0	2	3	3	1	1	4	8
		NOS	0	0	1	2	0	1	4	12
	Foci of cellular alteration	Any	43	45	40	48	48	46	43	50
		Basophil	19	30	26	35	46	36	31	35
		Clear cell	40	42	37	46	25	38	38	35
		Eosinophil	3	5	10	17	1	6	13	22
		Mixed cell	0	0	0	2	0	1	0	4
	Spongiosis hepatis		37	36	45	55	7	19	28	42
	Peliosis hepatis		12	11	7	12	8	14	15	15
	Necrosis focal, central or peripheral		6	6	9	7	9	6	12	10
	Fatty infiltration	Peripherolobular	47	38	32	42	42	38	33	44
		Centrilobular	5	4	10	11	3	2	6	6
		Diffuse	5	4	4	5	2	2	4	5
		Single cell or focal	18	18	16	3	22	21	20	9
	Lymphoid infiltration		37	16	9	20	17	19	8	17
	Bile-duct proliferation		61	50	44	50	48	40	39	41
Lung	Focal hyperplasia		0	1	2	3	0	0	0	1
	Foam cells		18	8	10	14	10	15	13	4
	Pneumonitis		39	34	21	33	35	24	27	30
Nasal cavity	Inflammation		20	36	47	52	9	35	50	42
	Atrophy of olfactory epithelium		7	21	48	58	0	30	51	56
	Hyperplasia	Basal cells	10	16	28	20	6	30	25	27
		Glands	0	7	21	44	0	16	37	39
		Goblet-cell	1	1	4	9	2	5	4	7
		Olfactory epithelium	0	13	47	46	2	26	41	54
		Focal	2	2	2	1	0	1	0	2
	Metaplasia	Septum	3	7	6	16	2	15	16	9
		Lateral wall	0	1	2	8	3	6	15	12
		Elsewhere	0	0	0	4	0	0	0	0
Pancreas	Focal hyperplasia of exocrine cells		3	0	1	7	0	0	0	1
	Giant islet		1	0	1	1	1	1	0	2
Trachea	Inflammation		3	2	1	3	1	0	4	1
	Epithelial hyperplasia		0	0	1	0	0	0	0	0

*NB. Sections examined microscopically from only a limited list of macroscopically normal tissues in terminally-killed rats of the low-dose and mid-dose groups (see text).

they are lined (i.e. respiratory or olfactory). As shown in Fig. 6(A, B), the basal cell hyperplasia and adenomas were principally observed at levels I and II where most of the epithelium is of the respiratory type. By contrast, the hyperplasia of glands and the adenocarcinomas were mainly found at levels II to IV where olfactory type epithelium predominates. Most of the adenomas in the anterior part of the nose were pedunculated and appeared to have arisen from respiratory-type epithelium or submucosal glands. The tumour cells were cuboidal or low columnar, often forming microcyst. By contrast, the adenocarcinomas appeared to have arisen from olfactory epithelium or from submucosal glands and many were poorly differentiated. Some of the adenocarcinomas extended into more than one level of sectioning. In only two instances were adenocarcinomas found in association with olfactory epithelium as far forward as level II. No tumours of either kind were found in the nasal cavities of control animals.

The morphology of the adenomas was totally different from that of the adenocarcinomas. Because of this difference in morphology, the different localization of the two types of tumours and the probability that they originated in different kinds of cells, it was concluded that it is unlikely that the adenomas were precursors of the adenocarcinomas or that the adenocarcinomas had arisen in pre-existing adenomas. Adenomas and adenocarcinomas should therefore be regarded as different kinds of neoplasm.

Statistical analyses of nasal cavity and liver tumour incidence

I. Nasal cavity

57 Rats developed adenomas in the nasal cavity; 49 rats in the main study, three rats in satellite group 2, and five rats in satellite group 3.

Altogether, 14 rats (all in main groups) developed adenocarcinomas of the nasal cavity. As these

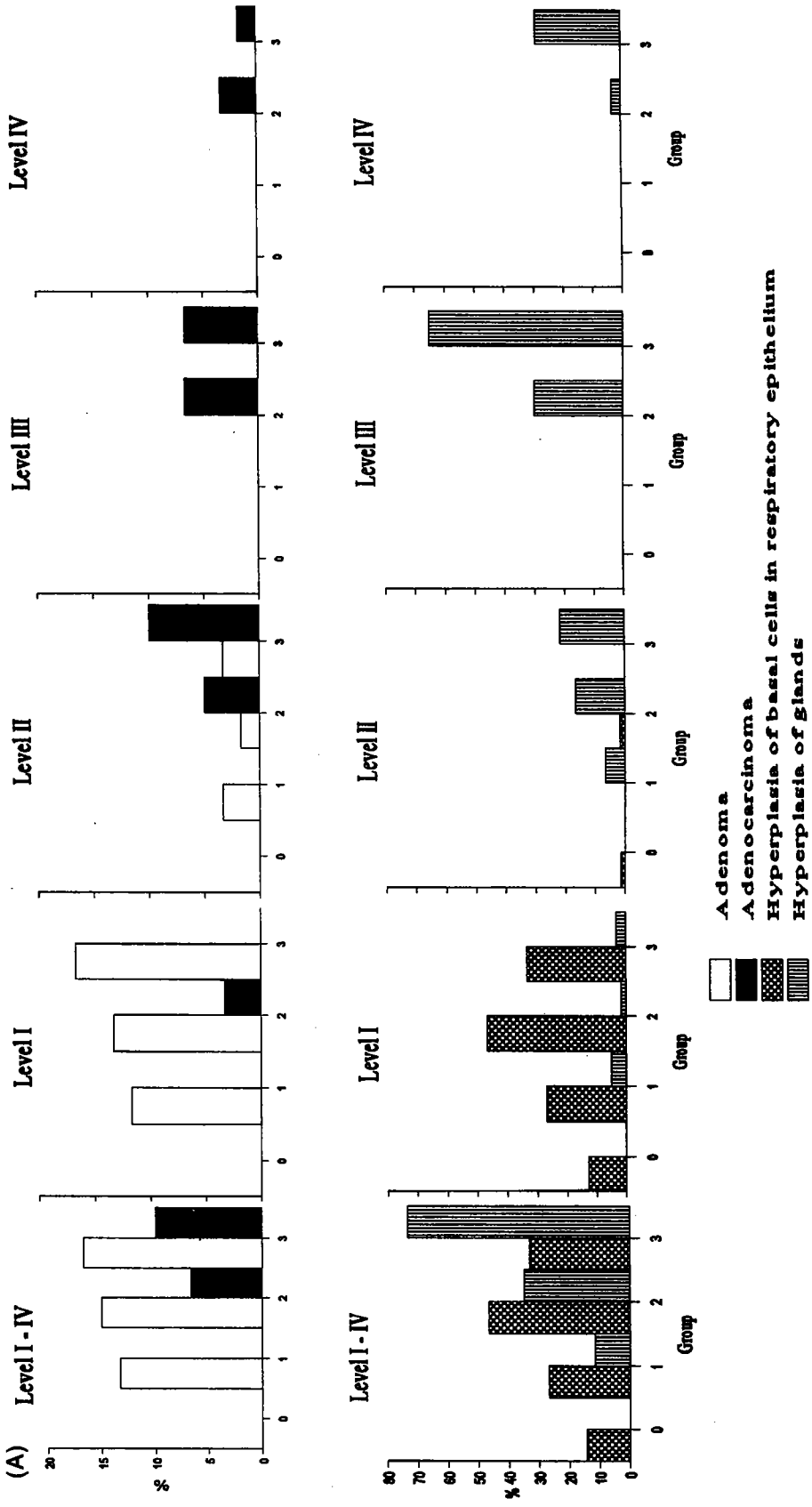
(A)
Level I - IV
Level I
Level II
Level III
Level IV
%
Group
Adenoma
Adenocarcinoma
Hyperplasia of basal cells in respiratory epithelium
Hyperplasia of glands

Fig. 6(A).

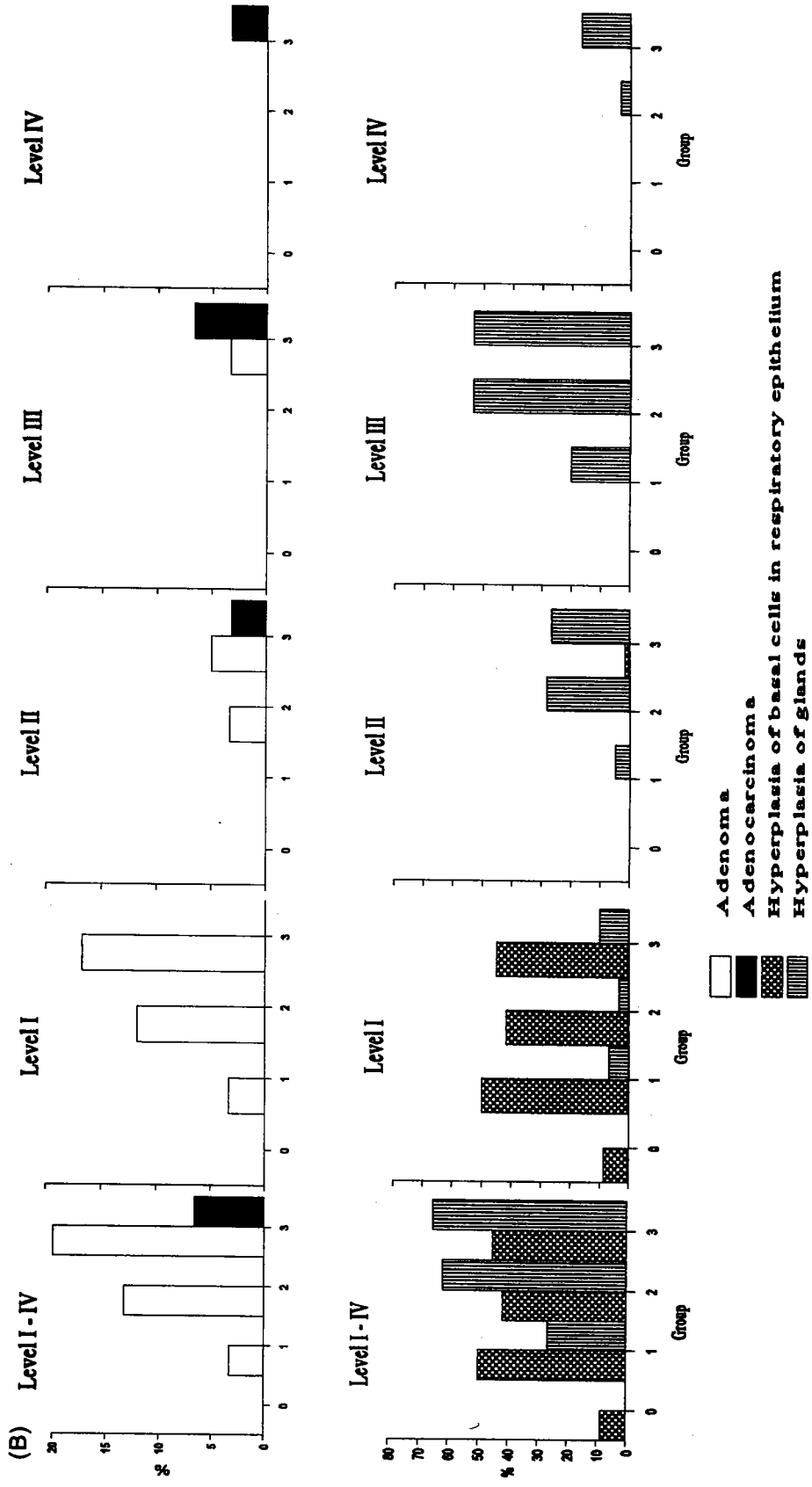

Fig. 6. Incidence of adenomas, adenocarcinomas, basal cell hyperplasia of the respiratory epithelium and hyperplasia of glands at different levels of sectioning in the nasal cavity of (A) male and (B) female rats killed at termination of the study.

tumours were pathologically different from the adenomas, they have been analysed separately (see Table 9). In animals continuously exposed until they died or were killed (including satellite 3 animals that died before 18 months, significant positive trends were seen in both sexes for both adenomas and adenocarcinomas. The effect on the incidence of adenomas was clear at all three exposure levels for males and at 10 ppm and 20 ppm for females. The effect on adenocarcinomas was evident for males at 10 ppm and 20 ppm and marginally for females at 20 ppm. An analysis of all 800 animals of the main and three satellite groups also showed significant trends in each of the sexes, both for adenomas and adenocarcinomas. The incidence of nasal cavity tumours in satellite 3 animals was less, but not significantly so, than that in continuously exposed rats.

II. Liver (Tables 8A, 9 and 11)

70 Rats developed hepatocellular carcinomas: 65 rats in the main study, and five in satellite 3.

All these liver tumours were considered to be incidental findings at necropsy and not to have contributed to death, so the Peto *et al.* (1980) method for incidental lesions is appropriate for their analysis.

1. The first analysis (see Table 9) was confined to all animals that were continuously exposed until they died or were killed, and included three satellite 3 animals that died or were killed before 18 months. There was a significant trend in both sexes for hepatocellular carcinomas. The effect was evident in all exposed groups in males and in the 10 ppm and 20 ppm groups in females.
2. A second analysis included satellite 3 animals that survived for more than 18 months. In other words, all 800 animals were included in this analysis. There was a significant trend in both sexes for hepatocellular carcinomas, and the effect was evident in males at 5 ppm and 20 ppm, and in females at 10 ppm and 20 ppm.
3. A third analysis (see Table 11) was based only on exposed rats that survived for more than 18 months, and compared the risk of liver tumour development in rats that were exposed continuously (main study) or not exposed (satellite 3 study). As can be seen, the incidence in satellite group 3 was significantly less than that in continuously exposed rats ($n = 5$, $E = 10.12$). Thus, cessation of exposure at 18 months with survival for up to 2 yr was associated with a significantly less than expected incidence of hepatocellular carcinomas compared with continuous exposure for up to 2 yr.

Study B

Analysis of concentrations of NVP, relative humidity and temperature

Monitoring, using the total hydrocarbons analyser, showed that the target concentration of 45 ppm NVP was closely achieved with mean concentration for the 3-month period being 45.4 ppm $\pm$ 0.84 (SD). Mean air temperature ranged between 21.4 and 24.5°C and mean relative humidity remained close to 53%.

Body weight

Figure 7 illustrates the effects of exposure to NVP on body weight during the 3-month exposure period. During the first 2 days of exposure to NVP, group 2 rats actually lost weight. However, thereafter, they gained weight more rapidly than the controls.

Clinical signs

The behaviour and appearance of both the exposed animals and the control animals remained normal throughout the study.

Survival

No animals died during the first 3 months of the study, that is, during the exposure period. Between 3 months and the termination of the study at

Table 11. Observed *v.* expected incidence of hepatocellular carcinomas in Satellite 3 animals exposed for 18 months and observed for up to 2 yr and main group animals that survived for 18 months or longer*

		Males		Females		Sexes combined	
Continuous exposure		Yes	No	Yes	No	Yes	No
Group 1 (5 ppm)	O	6	2	2	0	8	2
	E	6.81	1.19	1.58	0.42	8.40	1.60
	p		NS		NS		NS
Group 2 (10 ppm)	O	5	0	6	0	11	0
	E	4.39	0.61	4.95	1.05	9.35	1.65
	p		NS		NS		NS
Group 3 (20 ppm)	O	17	1	25	2	42	3
	E	15.56	2.44	22.57	4.43	38.14	6.86
	p	NS			(+)		+
Groups 1–3	O	28	3	33	2	61	5
	E	26.77	4.23	29.11	5.89	55.88	10.12
	p		NS		+		+

*= assuming that all tumours were incidental findings at necropsy; O = observed; E = expected;
NS= non-significant; + = $P < 0.05$.

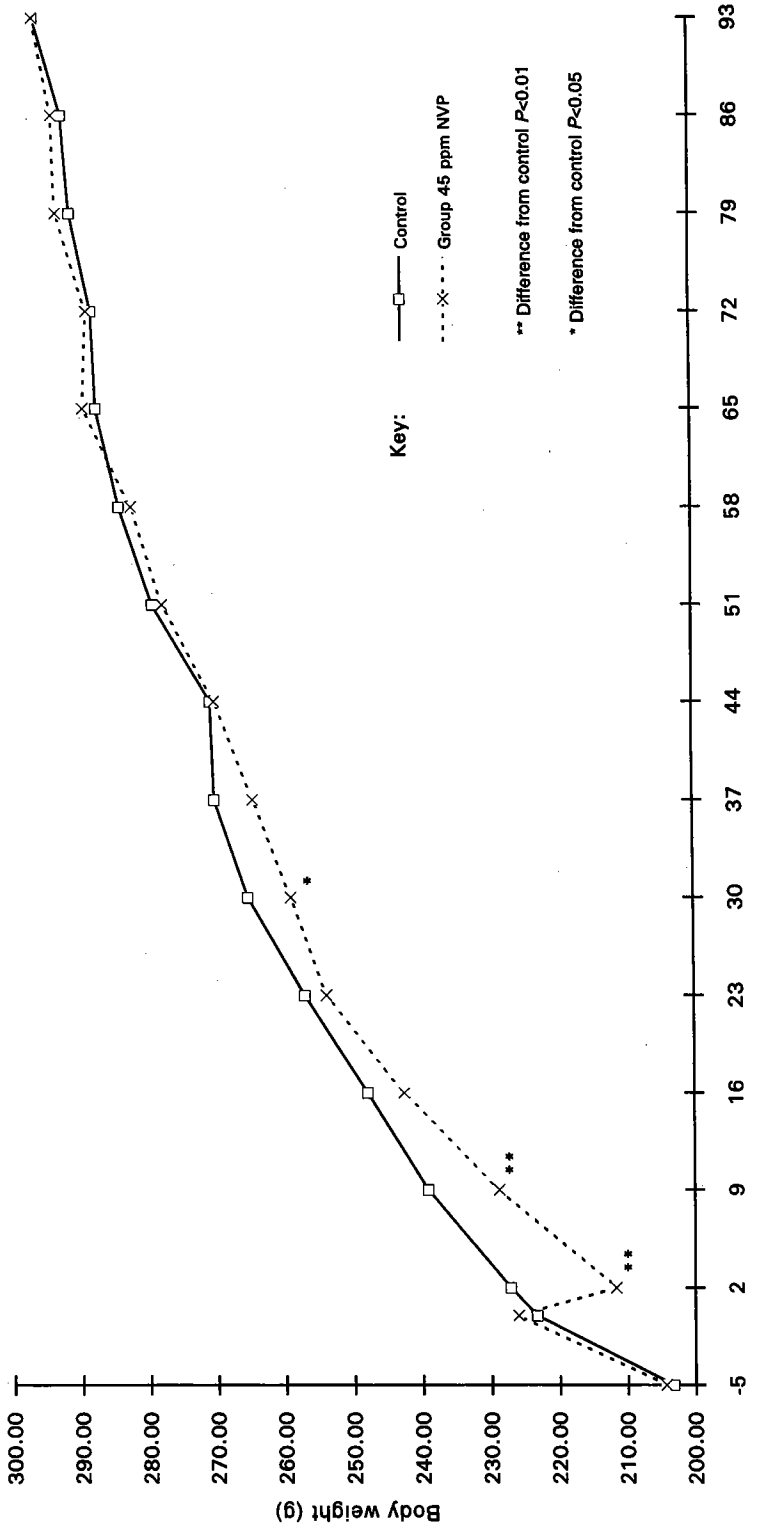

Fig. 7. Study B: effect of exposure to NVP on body weight in female rats.

Table 12. Survival of female rats in study B

Week	Interim or terminal sacrifice		Interim deaths (found dead or moribund)		Total deaths	
	Group 0	Group 2 (10 ppm)	Group 0	Group 2 (10 ppm)	Group 0	Group 2 (10 ppm)
7	5	5			5	5
14	10	10			10	10
14–26			0	0	0	0
26–39			1	0	1	0
39–52	10	10			10	10
52–65			1	1	1	1
65–78			1	3	1	3
78–91			3	1	3	1
91–104			5	3	5	3
104	4	6			4	6
Total	29	31	11	8	0	39

24 months, the numbers of animals that died or were killed in a moribund state was similar in the exposed and control groups (see Table 12).

Clinical chemistry

At all four sampling times (7 wk, 14 wk, 12 months and 24 months after the 3-month exposure period), liver γ-GT was significantly higher in NVP-exposed animals than in the controls. However, no difference between the two groups was found in the case of plasma γ-GT. Liver GSH was slightly, but not significantly, higher at 7 wk in the exposed animals. At 14 wk the elevation was statistically significant and remained so until the termination of the study. These various findings are summarized in Table 13.

Histopathological findings in the liver

The liver was the only tissue subjected to microscopic examination in study B.

In control rats killed 13 wk after the start of the exposure period, no abnormalities were found and there was virtually no glycogen present. In exposed rats killed at this time, centrilobular hepatocellular enlargement was seen. The enlargement was associated with the accumulation of coarse glycogen granules. Also present in these animals were foci/areas of hepatocellular change characterized by the absence of glycogen.

In control rats killed at the end of the exposure period (i.e. at 3 months), variable amounts of glycogen were seen but no pathological changes. By contrast, foci/areas of proliferative hepatocellular change, mainly composed of eosinophilic and/or clear cells, were seen in the NVP-exposed animals. Also, a basophilic focus was observed in one of the exposed rats.

In control rats killed 9 months after the end of the exposure period (i.e. 1 yr after the start of the study) small dark subcapsular foci were seen at necropsy. Some of these were depressed. Microscopically these lesions consisted of blood-filled sinuses and were diagnosed as sinus ectasia. Also, one control rat exhibited foci of cells some of which were clear and some eosinophilic. A few of these foci stained positively for glycogen in sections stained with PAS. Sinus ectasia was also seen in rats that had been exposed to NVP for 3 months and then observed without further exposure for 9 months. In addition, in these rats, foci consisting of clear or eosinophilic cells similar to those seen at the end of the exposure period were observed. Most of them stained positively for glycogen.

The findings in the livers of NVP-exposed and control rats that died or were killed between 12 and

Table 13. Study B—γ-GT activity in plasma (μkat/litre) and liver homogenate (μkat/g protein), and concentration of glutathione (GSH) (μmol/g) in the liver tissue of rats after exposure to 45 ppm *N*-vinylpyrrolidone-2 for 3 months and observation for 21 months

Test group (dose)		γ-GT in liver homogenate				γ-GT in plasma				GSH in the liver tissue			
		Sampling				Sampling				Sampling			
		1	2	3	4	1	2	3	4	1	2	3	4
0 (0 ppm)	N	5	10	10	4	5	10	10	4	5	10	10	4
	M	0.077	0.037	0.021	0.059	0.055	0.013	0.031	0.026	4.58	4.96	5.27	6.41
	SEM	0.018	0.006	0.002	0.020	0.013	0.002	0.008	0.003	0.32	0.21	0.17	0.29
2 (45 ppm)	N	5	10	10	6	5	10	10	6	5	10	10	6
	M	0.499**	0.474**	0.065**	0.489*	0.046	0.023	0.023	0.219	5.22	5.97**	6.55**	7.62*
	SE M	0.058	0.052	0.010	0.107	0.021	0.006	0.004	0.075	0.46	0.17	0.26	0.31

N = number of animals sacrificed as planned; M = mean; SEM = standard error; *significance ≥ 95%; **significance ≥ 99%.
Sampling 1 = sampling 49 days after start of administration (administration period).
Sampling 2 = sampling 99 days after start of administration (administration period).
Sampling 3 = sampling 12 months after start of administration (observation period).
Sampling 4 = sampling 24 months after start of administration (observation period).

Table 14. Study B—Histopathological findings in the livers of rats that died or were killed between 12 and 24 months of the study or were killed at 24 months

Group	**1**	**2**
Exposure to NVP during first 3 months (ppm)	0	45
No. of animals examined	15	15
Non-neoplastic changes		
Foci and/or areas of hepatocellular alternation (clear-cell or eosinophil)	9	8[1]
Focal cirrhosis-like metaplasia	0	2
Spongiosis hepatis	1	2
Focal telangiectasia	6	5
Bile-duct proliferation and fibrosis	9	9
Bile-duct cyst(s)	2	1
Liver-cell enlargement (cloudy, hydropic, vacuolar, hypertrophy)	0	6
Parenchymal necrosis (focal/multifocal)	5	2
Focal fatty degeneration	3	2
Peripheral fatty degeneration	1	0
Extramedullary haemopoiesis	4	0
Leukosis	1	0
Neoplastic		
Neoplastic nodule(s)	0	2[2]
Well-differentiated hepatocellular carcinoma	0	2[2]
Haemangiosarcoma	1	1

[1]Four of these animals also had hepatocellular neoplasms.
[2]Both in terminally killed rats.

24 months or were killed at termination of the study at 24 months are listed in Table 14. This table also includes data for four rats that died or were killed between 26 and 52 wk. In the case of non-neoplastic lesions, with the exception of liver cell enlargement (higher incidence in the NVP-exposed rats), and fatty infiltration (higher incidence in the control rats), the incidences of liver lesions were similar in the two groups. However, in the case of neoplastic lesions involving hepatocytes, a treatment-related effect was seen. Two terminally killed NVP-treated rats exhibited well-differentiated hepatocellular carcinomas and two had benign neoplastic nodules. In comparison, none of the control rats exhibited any neoplasm of hepatocellular origin. One treated rat and one control rat had a haemangiosarcoma arising in the liver.

In summary, 3 months' exposure to NVP predisposed the early appearance of foci/areas of mixed clear-cell eosinophilic hepatocellular change similar to those described by Stewart *et al.* (1980). These lesions did not apparently regress or disappear after the cessation of exposure to NVP. On the other hand, as the untreated control rats grew older, they too exhibited an increasing incidence of such lesions. Late in the study, four of the NVP-exposed rats but none of the controls were found to have developed liver cell neoplasms. Thus, two out of the six NVP-treated rats killed at the termination of the experiment at 2 yr exhibited neoplastic liver lesions. Although the numbers are small, it is reasonable to conclude that the development of liver cell tumours represented a treatment-related change, particularly since liver-cell tumours were also observed in study A.

DISCUSSION

The findings in study A point to there being two main target sites for the toxicity of NVP: the upper respiratory tract and the liver. Both targets were also identified in subacute/subchronic studies in rats and mice (Klimisch *et al.*, 1997).

The effects of long-term exposure to NVP on the nasal epithelium (including inflammation, atrophy of olfactory epithelium, basal cell hyperplasia, hyperplasia of submucosal glands, goblet cell hyperplasia, hyperplasia of olfactory epithelium, epithelial metaplasia, adenomas arising in respiratory-type epithelium or submucosal glands and adenocarcinomas arising in olfactory epithelium) and those on the larynx (inflammation, epithelial hyperplasia and neoplasia) constitute cascades of events attributable to direct contact between NVP and the epithelium in question. Insofar as NVP has been found to be non-genotoxic in a battery of *in vitro* and *in vivo* standard tests for mutagenicity and clastogenicity, it appears that the tumours that arose did so as a non-specific mechanism through continuously increasing cell proliferation processes as a consequence of repeated necrosis and regeneration. In recent years, it has been increasingly recognized that mutagens are formed endogenously during the conversion of ordinary foodstuffs to energy within body cells (Ames, 1983). Mutagens so produced damage cellular DNA. However, under normal conditions most of this damage is successfully and efficiently repaired. But repair may be impeded under conditions of high cell turnover rates as exist in repeatedly irritated tissues where there are high rates of cell death, mitosis and cell regeneration (Ames and Gold, 1990; Cohen and Ellwein, 1990). This may explain the neoplastic effects noted in the present study (study A) of neoplasms in the nose and larynx in response to highly irritating concentrations of NVP. Especially in the nasal mucosa, the typical findings for the necessary steps towards tumour formation could be demonstrated, namely necrosis and atrophy, hyperplasia due to regenera-

tive cell proliferation, benign and finally malignant neoplasia.

The effects of NVP on the incidence of non-neoplastic and neoplastic liver cell changes were not unexpected in the light of the findings in the earlier short-term studies (Klimisch *et al.*, 1997). However, the negative results of a battery of tests for genotoxicity (Greim, 1994; VCI, 1992) strongly suggest that liver tumour development in response to exposure to NVP does not involve a genotoxic mechanism. A possible explanation for the hepatocarcinogenicity may also be a continuous stimulus of cell replication by NVP either because of liver toxicity or by a receptor-mediated mechanism. On the other hand, it cannot be excluded that NVP is metabolically activated in the liver to a reactive short-lived proximal carcinogen, which, for whatever reason, was not detected in any of the various mutagenicity tests carried out with NVP. Therefore, the mechanisms underlying the hepatocarcinogenic effects of NVP remain to be elucidated. The fact that no adverse effects on tumour incidence was seen in any tissue other than the liver and the epithelia of the upper respiratory tract is consistent with the conclusion that if metabolic activation occurred in the liver, then the carcinogenic metabolite thereby formed was not transported through the bloodstream to other tissues.

Activation of γ-glutamyltransferase (γ-GT) has long been known to be associated with increased risk of hepatocarcinogenesis in laboratory rats and mice (Fiala and Fiala, 1973; Fiala *et al.*, 1976; Hanigan and Pitot, 1982 and 1985; Lans *et al.*, 1982; Sulakhe and Lau, 1987). Clearly, NVP fits into this pattern. However, the consensus of opinion seems to be that increased levels of γ-GT constitute simply a marker of exposure to a hepatocarcinogen and that γ-GT activation is not an integral part of the carcinogenic process.

In the earlier shorter-term studies on NVP, effects on various haematological parameters were seen (Klimisch *et al.*, 1997). Parameters relating to erythrocytes and platelets were the most consistently affected. In the presently reported 2-yr study effects on red blood cells (polychromasia, microcytosis and anisocytosis) were seen in satellite group animals of both sexes killed at 1 yr, whereas, after exposure for 2 yr, significant differences between treated and control groups in these parameters were only evident in females. In neither sex was there at any time any evidence of more serious haemotoxicity. Effects on the blood did not increase as the period of exposure to NVP was prolonged. The effects on blood cells indicate that NVP and/or its metabolites reached the bone marrow. This being so, it is noteworthy that the results of the *in vivo* mouse micronucleus test were negative (Greim, 1994).

In study B, an adverse effect on body weight seen during the 3-month period of exposure to 45 ppm NVP was no longer evident 31 wk after the end of exposure. Also, throughout the recovery period of 21 months following the 3-month exposure period, there were no differences between control and exposed animals in respect of clinical signs or survival. On the other hand, the effects of exposure on the liver did not reverse. Thus, γ-GT activity in liver homogenates remained persistently higher in exposed animals than in controls. Also, GSH levels in the liver remained high throughout the 21-month post-exposure period. However, the relevance of these effects to liver toxicity and carcinogenicity is unclear. Histologically-evident foci/areas of hepatocellular change present after 3 months of exposure to NVP did regress, but did not disappear during the subsequent 21-month exposure-free observation period. On the contrary, some of the animals exposed for just 3 months were found to have liver cell tumours when they were killed 21 months later, whereas none of the untreated controls had such tumours. The findings in this study, therefore, are consistent with the view that liver damage caused by NVP during a 3-month exposure period persists and may progress to neoplasia during a subsequent 21-month treatment-free period.

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