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THE REPEATED DOSE TOXICITY OF A SMOKE CONTAINING DISPERSE BLUE 180, AN ANTHRAQUINONE DYE MIXTURE

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Summary

This study was designed to assess possible health risks posed by repeated exposure to "working" concentrations of a smoke obtained from a pyrotechnic composition containing Disperse Blue 180. Groups of mice rats and guinea pigs were exposed to the test material 1h/d, 5d/week for 42 weeks at three different concentrations in a static chamber. Controls were exposed to air inside the chamber. After the last exposure, the animals were observed for 10 months. The test material caused a reduction in growth relative to the controls during the exposure period. A variety of incidental findings was seen in both decedents and survivors, but organ specific toxicity was generally confined to the respiratory tract. The most important of these findings was a statistically significant increase in the frequency of alveologenic carcinoma in the high dose group mice, when compared with the controls ($P < 0.05$). A variety of inflammatory changes was seen in the lungs of all species and some appeared to be treatment-related.

The aetiology of the excess of mouse lung tumours is discussed and it is pointed out that certain other anthraquinone dyes have been shown to be mutagenic and, in one case, carcinogenic in experimental animals.

Introduction

Coloured smokes have a variety of uses, both civilian and military. The former include the production of flares and fireworks, whilst military uses include battle-field training and firefighting. Generally such smokes are made by igniting a pyrotechnic composition, which usually consists of lactose and potassium chlorate together with a variety of dyes. In some cases more than one dye is employed [1,2] and the smoke used in the present experiment contains a proprietary mixture of anthraquinone dyes, collectively known as Disperse Blue 180, a dark blue powder supplied by Holliday Dyes and Chemicals, Huddersfield, U.K. (Table 1).

The inhalation toxicity of anthraquinone dyes has not been widely investigated. Two studies of inhaled coloured smokes in laboratory animals [1,2] have shown that organ specific toxicity is generally confined to the respiratory tract, the most consistent histological change being an extremely conspicuous

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TABLE 1

Composition of pyrotechnic mixture

Compound	wt.%
Lactose	23
Potassium chlorate	26
Zinc oxide	3
Disperse blue 180*	48

*This is a proprietary mixture containing anthraquinone dye(s) produced by Holliday Dyestuffs and Chemicals, Huddersfield, Yorkshire, U.K. It has no chemical constitution number.

macrophage response particularly in rats [1,3]. This finding, from studies on a smoke containing three dyes, demonstrated the retention in the lungs of one of them, the dye 1,4-ditoluidinoanthraquinone, Solvent Green 3, chemical constitution number 61,565 [4]. Similar accumulations of alveolar macrophages were noted by Sun et al. [5] after exposure of rats to a mixed aerosol of Solvent Green 3 and a quinoline dye, Solvent Yellow 33, chemical constitution number, 47,000 [4]. Although, macrophages were significantly more prevalent in a study of a smoke containing a mixture of the anthraquinone dye 1-amino-2-methyl-anthraquinone, Disperse Orange 11, chemical constitution number 60,700 [4] and Solvent Yellow 33, massive macrophage infiltration was not seen [2]. Thus there is experimental evidence of a considerable difference in degree of response between these two anthraquinones. It is likely that the difference is related to the very slow clearence of Solvent Green 3 from the lungs noted in guinea pigs by Marrs [3] and in rats by Sun et al. [5].

The other problems that have been associated with anthraquinone dyes, include, in the case of Disperse Orange 11, positive results in a carcinogenicity bioassay [6–8]. Additionally this dye was found to be mutagenic in the Ames test, using *Salmonella typhimurium*, strain TA 1537 R+, with or without S9 activation [2]. Further, a mixture of Solvent Green 3 and Solvent Yellow 33 was positive in Ames strains TA 102 and TA 104, with and without S9 activation [9].

Unlike the situation with military white smokes, where, particularly in the case of zinc oxide/hexachloroethane smoke, reports of lung damage are very frequent [10–14], with coloured smokes, acute toxic effects in humans seem exiguous. Since there is the potential for exposure, particularly of military personnel, as part of our continuing programme on the toxicity of military smokes, we have investigated a further smoke, containing anthraquinone dyes. In view of the tests *in vitro* on other anthraquinones, described above, we report the results of an Ames test on the material Disperse Blue 180.

Method

Animals

Four hundred 35-days old Porton-strain female mice, of mean weight 23 g, 200 35-d old Porton Wistar-derived female rats, of mean weight 105 g, and 192 37-d old Dunkin–Hartley female guinea pigs, of mean weight 288 g, were supplied by the Animal Breeding Unit, CDE Porton Down. The animals were randomly allocated into groups of approximately equal sizes, designated controls, low, medium and high. In the case of the mice one death after randomization, but before the start of exposure, caused the size of the medium group to be 99. In the rats, group size was 50 in all cases, whilst the size of the dose groups of guinea pigs was 48. Groups of animals were then subdivided: in the case of the mice, this was into subgroups of ten to a cage (nine in one case), whilst the rats were subdivided into groups of five; the guinea pigs were similarly divided into groups of six to a cage. Special precautions were not taken to prevent huddling, since this phenomenon appears not to affect the inhaled dose of toxicant at least in the case of rats [15]. Between exposures, the animals were housed in special accommodation near the inhalation unit, and then, after the last exposure, transferred to permanent accommodation.

Generation of the smoke

The pyrotechnic mixture was made at ROF Glascoed, now Royal Ordnance PLC, Gwent, Wales, U.K. This mixture (Table 1) was in the form of thick washers of mean weight 1 g. They were ignited on electically heated nichrome wire, the concentration of the smoke being varied by using different numbers of washes. The resulting blue smoke was mixed in a static 10 m^3 chamber with high velocity air jets.

Exposure

The animals were exposed to the freshly generated smoke, 1h/d, 5d/week, until they had experienced 200 exposures (i.e. 42 weeks, Table 2). Corresponding groups of each species were exposed together, starting with exposure to air within the chamber, of the controls and ending with the high dose group. The anticipated concentrations of the smoke were 50, 160 and 500 mg m^{-3}, the last being the expected maximum tolerated concentration. The high concentration was about 3.25 times the medium, which was in turn about 3.25 times the low concentration. Because the concentration of smoke fell during exposure, fresh washers of the smoke composition were ignited, to maintain the desired concentration. During the half-hour interval, between exposure of the various dose groups, the chamber was thoroughly cleaned: at the end of the high dose exposure, the chamber was well-washed twice.

The smoke was sampled by drawing air through absolute glass fibre filters. Six samples of 10 min duration, 8 samples of 5 min and 7 samples of 2 min

TABLE 2

Exposure doses and concentrations of smoke during the study

Test	Daily conc.* mg m^{-3}, $\bar{x} \pm$ SD	Total number of exposures	Total dose* (*Ct*, concentration × time) mg min m^{-3}
Control	0	200	0
Low	51.5 ± 5.9	200	618, 840
Medium	156.2 ± 10.5	200	1,874, 400
High	500.4 ± 25.5	200	6,004, 800

*Concentrations and dose of solid material (for analysis see methods section).

were taken in the low, medium and high dose exposures respectively. The impacted mass of smoke was weighed, allowing calculation of the concentration of solid material during exposure. The particle size of the smoke was determined using an Anderson particle sizer.

During the exposure period animals were examined for abnormal behaviour or ill-health. They were weighed weekly during exposure, and monthly during the ensuing 10 month observation period. The mean whole body weight of each group of animals relative to the corresponding control group was plotted against time. Animals showing signs of ill-health were killed using sodium pentobarbital: those alive at the end of the study (20 months after the start of exposure) were similarly killed. Animals killed or found dead were examined post-mortem. At autopsy, lungs, larynx, trachea, liver, kidneys, spleen, thymus, adrenal and thyroid glands, ovaries, heart, pancreas, oesophagus, stomach, small and large intestine were taken, as well as cervical lymph nodes in rats and guinea pigs. Any other organ showing any macroscopical abnormality was also processed. After fixation in neutral buffered formalin, sections 5 μm thick were cut and stained with haematoxylin and eosin. Other stains used in certain instances included Perls' Prussian blue method for haemosiderin as well as Van Gieson's stain [16]. Histological data were recorded and analysed using a PLACES data acquisition system, Apoloco Ltd, 90 King Street, Newcastle-under-Lyme, England, ST5 1JB, U.K. This package was run on a VAX minicomputer, Digital Equipment Corporation, Maynard, Mass., U.S.A.

Statistics

The frequency of histological changes in animals from the test groups was compared to the frequency in the corresponding control groups, using Fisher's exact test. Additionally, in the case of the mouse alveologenic carcinomas, a statistical test for a trend was performed [17]. This test was carried out using a linear dose scale and a logarithmic transformation thereof. Histological data

from the decedents were not statistically analyzed because the numbers were insufficient.

Ames test

The Ames *Salmonella typhimurium* test was carried out on the dye Disperse blue 180 as follows. Dye samples were dissolved and diluted in dimethylsulphoxide (Aldridge) to provide incremental concentrations in the range 0.195–100 μg/plate. The test was carried out as described by Ames et al. [18], three dye samples being tested in triplicate plates in the presence and absence of rat liver homogenate induced with Aroclor 1254 (S9), using the indicator strains, TA 1535, TA 1537, TA 1538, TA 98, TA 100 and TA 1537R+. All the strains were obtained from the laboratory of Dr. Bruce N. Ames except for TA 1537 R+, which was created by transfection of plasmid pKM101 from a donor strain of *Escherischia coli* as previously described [2]. Aroclor 1254 was kindly given by Monsanto Chemicals.

Results

Analysis of smoke

The concentrations of smoke attained were close to the intended concentrations (Table 2). The mass median diameter was very variable but always lay between 0.5 and 1 μm.

Growth

The test material had some effect on weight gain: in the mice and the rats, the relative weight of the test groups fell during exposure, without exception. A marked recovery occurred during the observation period in all the test groups of mice, and to a lesser extent, in the high dose group of rats. The weight of the test groups of guinea pigs, relative to the controls, fell rapidly during the early part of the exposure period, and thence recovered slightly.

Survival

In general there was no obvious dose-related effect on survival (Table 3). There was a considerable number of decedents amongst the mice, but far fewer in the rats. In the latter species the greatest number of early deaths was seen in the controls, whilst in the guinea pigs the smallest number of early deaths was seen in the high dose group.

Early deaths

In the mice histological changes were varied: many of the animals died with chronic murine pneumonia and/or acute pneumonitis. Macroscopically, it was observed that abdominal fat was sometimes stained blue in the test groups. Generally confined to the high dose group were pulmonary changes such as

TABLE 3

Cumulative mortality by months for each species during the study

Months		Exposure										Observation										End of study
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Mice	C	0	0	0	2	2	3	5	7	7	9	15	18	17	19	19	21	27	29	38	39	100
	L	0	0	3	3	4	4	5	6	6	6	7	8	9	12	14	15	17	20	24	29	100
	M	0	0	1	1	1	2	2	3	3	4	5	5	6	12	14	15	18	23	26	31	99
	H	0	1	2	5	5	5	6	6	6	6	7	8	9	13	15	19	22	25	31	33	100
Rats	C	0	0	0	0	0	0	1	1	1	1	1	1	3	3	4	7	8	8	9	10	50
	L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2	3	4	6	50
	M	0	0	0	0	0	0	0	0	0	0	0	0	1	4	4	4	4	6	6	7	50
	H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	3	3	5	50
Guinea Pigs	C	0	0	0	0	0	0	0	1	1	1	2	4	4	6	6	7	8	8	9	11	48
	L	0	0	0	0	0	1	2	4	4	4	5	5	5	5	5	5	6	6	7	9	48
	M	0	0	0	0	0	0	2	2	2	3	4	6	6	7	7	7	7	7	9	11	48
	H	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2	3	48

infiltration with macrophages containing granules and in a few cases foam cells were seen. Lung abscesses, chronic interstitial nephritis and fatty change in the liver were frequently seen in all dose groups. Tumours seen included alveologenic carcinoma, observed in 2/39, 2/29, 5/31 and 1/33 cases in the control, low, medium and high does respectively. Lymphosarcoma, usually thymic, was seen in three controls, one low, three medium and three high-dose decedents. Other tumours occurred in single instances only, and included a skin appendage tumour in a low dose mouse decedent. In the decedent rat nephropathy was very common. Tumours were only seen in the controls and the high dose groups, the most common being fibroadenoma of the mammary gland. Centrilobular hepatic necrosis was seen in one animal each from the low and high dose group animals. Some alveolar infiltration with macrophages containing granules was seen in decedents from the high dose groups of rats and this change was also observed amongst the guinea pigs. In one each of the control and low dose guinea pigs, marked bile duct proliferation was seen, with, in the latter case, considerable bile stasis. In a medium dose group animal a cholangiocarcinoma was seen, and an alveolar-cell carcinoma of the lung was seen in another animal from this group.

Survivors

Histological findings

The most important histological finding in the mice was a high frequency of alveologenic carcinoma in the top dose group (Table 4). Using the data from

TABLE 4

Mice surviving to the end of the study: changes seen in the respiratory tract (selected)

Organ and Finding	Dose group[a]			
	Control (61)	Low (71)	Medium (68)	High (67)
Trachea				
Chronic inflammatory change	17	20	8[b]	1[b]
Larynx				
Chronic inflammatory change	47	47	47	37[b]
Lungs				
Alveologenic carcinoma	6	6	11	15[c]
Alveolar macrophages	0	0	2	0
Peribronchial macrophages	5	3	3	9

[a]Figures in parentheses are total number of animals autopsied.
[b]$P < 0.05$ — finding significantly less frequent in test group than control.
[c]$P < 0.05$ — finding significantly more frequent in test group than control.

all the test groups and the controls the statistical test for a trend against dose was significant at $P = 0.05$, but against log dose it was not significant at this level. The other change in the mouse lungs, which was probably related to the test material was macrophage infiltration; this was statistically and significantly more prevalent in the high dose group than the controls. Most of the alveolar macrophages contained brown granules and in a few instances foam cells were present. Peribronchial macrophages were also observed. Other changes seen in the mouse lungs were infrequent or clearly incidental (Table 4). Thus some degree of collapse and congestion was seen in the majority of cases. Rarely seen, and also unrelated to dose, was pleurial fibrosis. Tracheal chronic inflammatory changes were notably less common in the test animals of the medium and high groups than the controls.

In the extra-respiratory organs of the mice, the vast majority of the changes were not dose related (Table 5). Such changes included lymph node adenitis. Hepatic lymphocytic infiltration was usually periportal, but occasionally perivascular in distribution. Tumours seen, included fibroadenomas in mammary and salivary glands, an adrenal phaeochromocytoma and a papilloma of the skin. Tumours were also seen in the uterus and ovaries. No abnormality was seen in the gastrointestinal tract, i.e. oesophagus, stomach, duodenum, jejunum, ileum and colon.

As in the mice, dose related changes in the rat were found in the respiratory tract. Unlike the situation with the mice, none of these was neoplastic (Table 6). Macrophage infiltration was seen in a very high proportion of the lungs from the higher dosed groups. In the case, specifically, of foam cells (Fig. 1),

TABLE 5

Mice surviving to the end of the study: histological changes seen in extrarespiratory organs (selected; figures in brackets are total number of animals autopsied)

Organ and Finding	Dose group			
	Control (61)	Low (71)	Medium (68)	High (67)
Mammary gland				
Fibroadenoma	1	0	0	1
Lymph nodes				
Infiltration with lymphosarcoma	0	1	0	0
Salivary gland				
Fibroadenoma	1	0	0	0
Thymus				
Infiltrated with lymphosarcoma	0	0	0	1
Heart				
Pericardial fibrosis	0	0	0	2
Liver				
Fatty change	16	19	27	11
Hepatocellular carcinoma	0	1	0	0
Nodular hyperplasia	5	4	9	5
Lymphocytic infiltration	2	3	3	1
Infiltration with lymphosarcoma	0	0	0	1
Pancreas				
Perivascular lymphocytic infiltration	0	0	0	1
Spleen				
Lymphoid hyperplasia	0	0	0	1
Infiltration with lymphosarcoma	0	1	0	1
Kidneys				
Infiltrated with lymphosarcoma	0	0	0	1
Adrenals				
Phaeochromocytoma	0	0	0	1
Thyroid				
Lymphomatous infiltration	0	0	0	1
Ovary				
Serous cysts	1	1	4	2
Leiomyoma	0	0	0	1
Granulosa cell tumour	0	0	1	0
*Uterus**				
Leiomyoma	1	0	1	0
Adenocarcinoma	0	0	1	0
Skin				
Papilloma	0	1	0	0

*Non protocol organs.

TABLE 6

Rats surviving to the end of the study: histological changes seen in the respiratory tract (selected; figures in brackets are total number of animals autopsied)

Organ and Finding	Dose group			
	Control (40)	Low (44)	Medium (42)	High (44)
Trachea				
Polymorph infiltration	1	0	0	0
Larynx				
Lymphocytic infiltration	1	1	0	0
Lung				
Fibrosarcoma	0	0	1	0
Foamy macrophages (alveolar)	1	0	0	11[a]
Granular macrophages (alveolar)	1	22[a]	42[a]	44[a]
Perivascular granular macrophages	0	2	31[a]	41[a]
Peribronchial granular macrophages	26	38[b]	40[a]	31
Generalised macrophage infiltration	5	1	3	2
Peribronchial lymphocyte infiltration	33	40	40	42
Perivascular lymphocytic infiltration	9	15	12	29[a]

[a] $P < 0.01$ — finding significantly more frequent in test group than control.
[b] $P < 0.05$ — finding significantly more frequent in test group than control.

the difference between the high and control groups was significant statistically ($P < 0.01$). Macrophages containing dark granules were frequently seen in the test groups (Fig. 2). These were often seen in the alveolar walls, and sometimes in the alveolar space. When the frequency of granular macrophages in the alveoli was statistically analyzed, all the test groups were significantly more often affected than the controls ($P < 0.01$, all cases). Perivascular granular macrophages were also significantly more frequent in the middle and high dose groups than the controls ($P < 0.01$), whilst peribronchial granular macrophages were significantly more prevalent in the low ($P < 0.05$) and middle ($P < 0.01$) dose groups than the controls. A significant difference between the frequency of this change in the high dose group and controls was not seen. Lymphocytic infiltration was also frequent: perivascular infiltration with lymphocytes was more frequently present in the high dose group than the controls ($P < 0.01$). Other changes in the rat lung appeared to be incidental, and included collapse and congestion in the majority of lungs. Changes were only rarely seen in the trachea or the larynx (Table 6).

Of the extrarespiratory organs examined, the heart, pancreas, oesophagus, stomach, jejunum, ileum and colon appeared normal in all cases (Table 7). In the liver fibrosis was seen in one or two cases from all groups. Nephropathy was seen in all groups and was common.

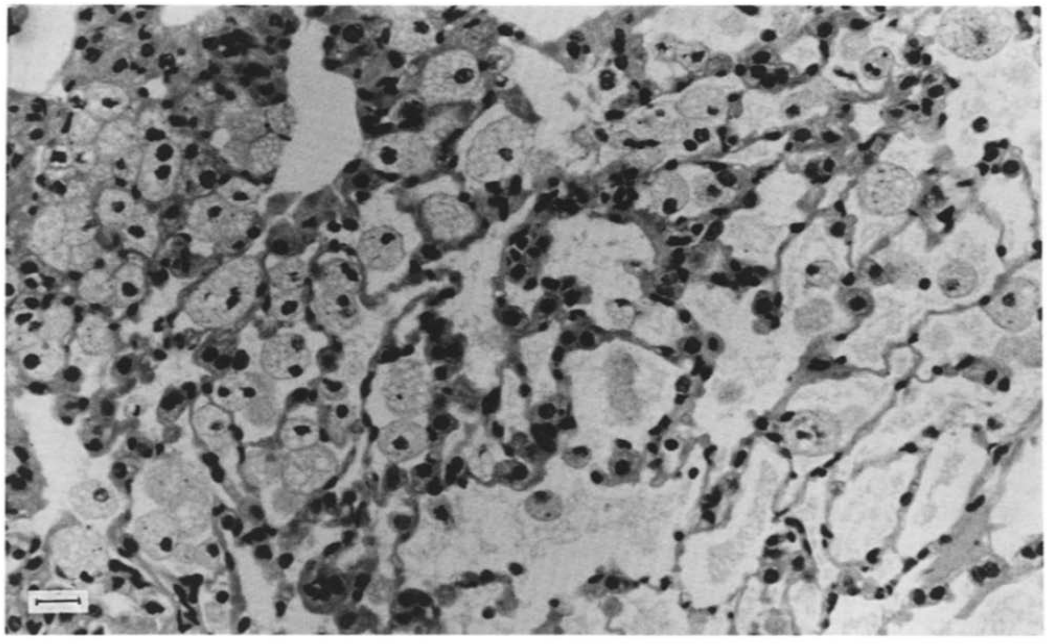

Fig. 1. Section of a lung from a rat dosed at the highest level, showing foamy macrophages (bar 20 μm).

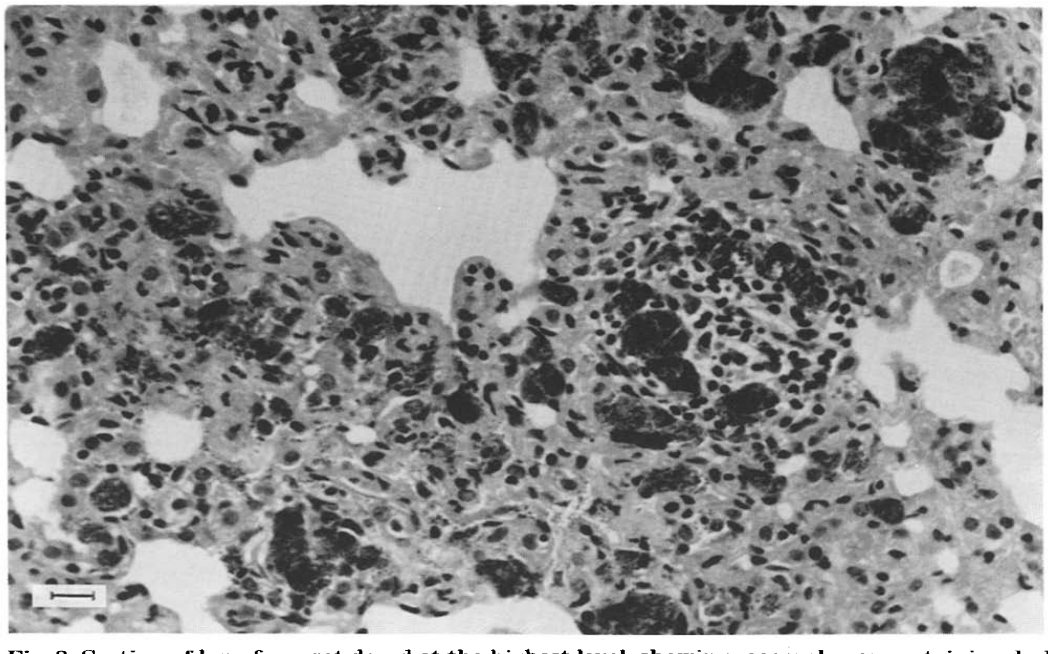

Fig. 2. Section of lung from rat dosed at the highest level, showing macrophages containing dark granules (bar 20 μm).

TABLE 7

Rats surviving to the end of the study: histological findings seen in extrarespiratory organs (selected; figures in brackets are total number of animals autopsied)

Organ and Finding	Dose group			
	Control (40)	Low (44)	Medium (42)	High (44)
Mammary gland				
Adenocarcinoma	0	1	0	0
Fibroadenoma	2	2	4	4
Fibrosarcoma	0	0	1	0
Lymph nodes				
Excess macrophages	0	0	1	0
Salivary gland				
Fibroadenoma	0	0	0	1
Thymus				
Lymphoma	0	1	0	0
Cystic change	5	6	3	3
Liver				
Kupffer cell tumour	1	0	0	0
Spleen				
Angioma	0	0	1	0
Kidneys				
Nephrocalcinosis	16	25	13	22
Nephropathy	25	34	25	28
Cortical lymphocytic infiltration	11	16	13	15
Adrenals				
Macrophage infiltration	1	0	0	0
Ovaries				
Serous cyst	2	1	3	0
Fibroma	1	0	0	0
*Uterus**				
Adenocarcinoma	0	0	0	1
*Pituitary**				
Adenoma	0	0	1	0
*Skin**				
Epidermal cyst	1	0	0	0
Hyperkeratosis	1	0	0	0

*Non protocol organs.

Neoplastic lesions were infrequently observed in the guinea pigs (Table 8). The main changes seen in the lungs were macrophagocytic or lymphocytic infiltration. Infiltration with macrophages containing granules appeared to be dose-related, alveolar granular macrophage infiltration being significantly more frequent in the middle and high dose groups than in the controls ($P<0.01$). Peribronchial granular macrophages were similarly distributed between the

TABLE 8

Guinea pigs surviving to the end of the study: histological changes seen in all organs (Figures in brackets represent total numbers of animals autopsied)

Organ and Finding	Dose group			
	Control (37)	Low (38)	Medium (37)	High (45)
Trachea				
Chronic inflammatory changes	37	34	28[a]	27[a]
Larynx				
Chronic inflammatory changes	21	16	14	14[a]
Lungs				
Alveolar granular macrophages	0	5	33[b]	42[b]
Bronchiolar granular macrophages	0	2	4	9[b]
Peribronchial granular macrophages	3	7	30[b]	42[b]
Peribronchial lymphocytic infiltration	24	35[b]	33[c]	40[c]
Perivascular lymphocytic infiltration	36	37	35	44
Mammary gland				
Fibroadenoma	0	0	1	0
Heart				
Pericardial lymphocytic infiltration	1	5	0	0
Liver				
Focal necrosis	0	1	0	0
Kidneys				
Nephrocalcinosis	34	29	29	29[a]
Thyroid				
Nodular hyperplasia	0	0	0	1

[a] $P < 0.05$ — finding significantly less frequent in test group than control.
[b] $P < 0.01$ — finding significantly more frequent in test group than control.
[c] $P < 0.05$ — finding significantly more frequent in test group than control.

groups, while bronchiolar granular macrophages were significantly more common in the high dose group only ($P < 0.01$). Whilst perivascular lymphocytic infiltration was common in all the groups, peribronchial lymphocytic infiltration was significantly more prevalent in all three test groups ($P < 0.01$, low vs. control; $P < 0.05$; middle and high vs. control). Foamy macrophages were seen in one instance only, this being a control animal. No abnormality was detected in the salivary glands, thymus, gut or spleen, whilst abnormalities were exiguous in the pancreas and adrenals. Serous cysts were very frequent in ovaries from all groups and nephrocalcinosis was frequently seen in the kidneys.

Ames test

The dye Disperse Blue 180 was mutagenic for the indicator strain TA 1537R+, and non-mutagenic for the strains TA 1535, TA 1537, TA 1538, TA

TABLE 9

Reversion of *Salmonella typhimurium* strain TA 1537R+ by Disperse Blue 180 (DB 180) in the presence or absence of Aroclor induced rat liver homogenate (S9-mix)

Dye conc. (μg/plate)	S9	Numbers[a] of reversions induced by DB 180		
		Test 1	Test 2	Test 3
100	+	1665	1692	1636
50	+	1323	1069	1354
25	+	1148	1040	1140
12.5	+	1083	849	1274
6.25	+	1036	523	830
3.125	+	664	596	690
1.56	+	716	596	848
0.78	+	617	450	804
0.39	+	489	460	442
0.195	+	358	263	352
0	+	366	291	313
100	–	1642	1932	1818
50	–	1293	1784	1830
25	–	1236	1402	1470
12.5	–	1115	1188	1262
6.25	–	840	936	764
3.125	–	741	660	728
1.56	–	593	644	560
0.78	–	506	566	440
0.39	–	435	444	478
0.195	–	297	148	410
0	–	344	268	448

[a]Numbers of revertants listed above reflect an average from the counts of three plates.

98 and TA 100. The response was not affected by the presence or absence of S9-mix (Table 9). The mutagenic response was observed at dye concentrations of above 6.25 to 12.5 μg/plate, a positive response being taken as a number of revertants 2.5 times that in the solvent control. The maximum concentration of dye did not cause toxicity in the indicator strains. The number of revertants in plates containing known mutagens was in the range expected.

Discussion

There was an effect on growth and while this was partially reversible, especially in mice, a no effect level could not be established. As far as longevity is concerned, there was no evidence of excess deaths in the dosed animals over the corresponding controls, indeed in the mice the mortality was highest in the

control group. Amongst the decedents, only respiratory tract changes, such as macrophage infiltration seemed to be dose related.

The majority of the changes observed in the survivors were predictable on the basis of previous studies or clearly incidental. Exceptions seemed confined to the respiratory tract. A finding in the study which gave cause for concern was the frequency of alveologenic carcinomas in the highest dose group of mice where the difference from the controls was significant ($P < 0.05$). In the study, these tumours were not separated into benign and malignant on morphological grounds as is sometimes recommended [19,20] and instead the criteria for histologial diagnosis of Stewart et al. [21] were used. They state firmly that the tumour should be regarded as malignant *ab initio*. The spontaneous frequency of this tumour is high in many strains of mouse [21,22] and it appears that the incidence is under genetic control [23]. Previous studies in this laboratory, have shown that the Porton mouse has a moderately high incidence [1,2,24]. Alveologenic carcinoma is not histologically similar to the common types of human lung cancer, nevertheless Shimkin and colleagues have applied this tumour to chemical carcinogenesis bioassay with satisfactory results [25,26]. Therefore, it must be concluded that the present study, while in no sense designed as a carcinogenesis bioassay, has provided evidence of experimental carcinogenicity in mice. Despite the marginal significance, this finding must be interpreted conservatively in view of previous mutagenicity and carcinogenicity results on anthraquinone dyes and the mutagenic response reported here. However the mutagenicity was dependent on the frameshift indicator strain TA 1537R+, a strain of particular sensitivity to anthraquinone dyes, and the substitution indicator strains, TA 1535 and TA 100, did not show a mutagenic response. Since the frameshift indicators, TA 1537, TA 1538 and TA 98, failed to demonstrate mutagenic potential, the positive response was based on a single Salmonella indicator, that is only infrequently used.

The most noteworthy non-neoplastic lung finding was the macrophage infiltration observed in the rats, and to a lesser extent in the other two species. This change, which was probably test material related, is reminiscent of the findings of Marrs et al. [1,2] and others [5].

Of the more noteworthy incidental findings, renal disease is common in all small laboratory animals and chronic interstitial nephritis is frequently seen in the Porton mouse [1,2,24] and in guinea pigs [27]. Similarly rat nephropathy, which is called by a number of names including nephrosis, glomerulonephrosis, chronic nephritis and chronic interstitial nephritis, is a common condition of unknown aetiology [28–31].

It is difficult to relate the concentrations used in the present study to those, to which humans are likely to be exposed. The reason for this is that these smokes are used in the open air and because of dispersal the concentration is highly variable. Over short periods, concentrations of the type received by the

high dose groups in the present study might be experienced by humans. Nevertheless the high dose, in terms of concentration × time (Ct) is considerably above any likely human exposure. Therefore the findings on survival suggest that the smoke would be relatively innocuous. Nevertheless, some caution is advised with this and similar smokes, in view of the mouse lung tumours and the mutagenicity result.

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