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# INHALATION TOXICITY OF A COLOURED SMOKE AND THE MUTAGENICITY OF ITS CONSTITUENT DYES, SOLVENT YELLOW 33 (CI 47,000) AND DISPERSE ORANGE 11 (CI 60,700) IN THE AMES TEST

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## Summary

The repeated dose toxicity of a smoke containing Solvent Yellow 33 and Disperse Orange 11 was studied in three species of small laboratory animal. Except in guinea-pigs, exposure did not affect survival but rate of growth was diminished in the test groups by comparison with corresponding controls. Organ specific toxicity was confined to the respiratory tract. Disperse Orange 11 was mutagenic in the Ames test for the Salmonella indicator strain TA 1537 R+ with or without S9 activation whilst Solvent Yellow 33 was marginally mutagenic to the same indicator strain, also with or without S9 activation. Neither dye was mutagenic for strains TA 98, TA 100 or TA 1537 irrespective of S9 activation.

## Introduction

Coloured smoke-producing pyrotechnic compositions have a variety of uses which range from the production of flares and fireworks, to their use in military situations. They generally consist of a pyrotechnic mixture together with a variety of dyes, designed to produce the desired colour.

The toxicity of a smoke generated by ignition of a pyrotechnic composition containing 2 dyes (Table 1) has been studied in the course of a programme of investigation of the toxicity of selected military smokes. The inhalation toxicity of a related smoke, containing 3 dyes has already been reported [1]. The present paper reports the inhalation toxicity of a smoke containing 2 dyes: additional substances in the pyrotechnic mixture are common to many military smoke compositions, both coloured and non-coloured.

Very little information is available on the biological effects of Solvent Yellow 33 [2-(2-quinolyl)-1,3-indanedione] with the exception of its skin-sensitisation properties [2,3]. The dye is closely related to the food dye Quinoline Yel-

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TABLE 1

Composition of pyrotechnic mixture used to generate the smoke

| Constituent* | % by weight |
|---|---|
| Lactose monohydrate | 25 |
| Potassium chlorate | 26 |
| Zinc oxide | 3 |
| Solvent Yellow 33; 47000[1] | 28 |
| Disperse Orange 11; 60700[1] | 18 |

[1]Society of Dyers & Colourists [4], Chemical Constitution Number.
*Ministry of Defence [13].

low, E 104 (Colour index, 47005, Society of Dyers and Colourists [4]). Nevertheless the two dyes differ chemically in one important respect, namely the possession by Quinoline Yellow of sulphonate groups, which cause the solubility characteristics of the two dyes to be quite different. As a result one cannot extrapolate the results of toxicity studies on Quinoline Yellow to Solvent Yellow 33. Notwithstanding these reservations it may be noted that the food dye is poorly adsorbed and non-toxic when given by mouth [5–8]. Quinoline Yellow would also appear to be non-toxic when given parenterally [9].

Disperse Orange 11 (1-amino-2-methylanthraquinone) is a dye which has been reported to be carcinogenic to rats in which it increased the prevalence of hepatocellular and renal neoplasms. In mice the main histological changes were in the renal tract, including glomerulonephritis and interstitial nephritis [10,11]. In the latter paper the authors speculated that the tumorigenic effects of Disperge Orange 11 might be caused by formation of an *N*-hydroxylated compound. A critical evaluation of Disperse Orange 11 [12] suggested that no data were available on reproductive or perinatal toxicology or on the mutagenicity of Disperse Orange 11. Furthermore the review concluded that the toxic potentialities of the dye had not been fully evaluated.

The study, described below, deals with the inhalation toxicity of the smoke in three species of laboratory animal. During the programme of investigation of the toxicology of the coloured military smokes, the mutagenic potential of their constituent dyes was evaluated, using the Ames test. The results of that test on the two dyes found in the smoke, used in the present study, are also reported here.

## Experimental

*1. Animals*

Four hundred 24-day old Porton-strain SPF female mice mean weight 15.30 g, 200 31-day old Porton Wistar-derived female rats mean weight, 80.1 g and

200 31-day old Dunkin–Hartley female guinea-pigs* mean weight, 229.5 g were supplied by the animal breeding unit, CDE. The animals were allocated randomly to equal sized groups designated controls, low, medium and high. The animals were divided into subgroups of five per cage. No special precautions were taken to prevent huddling since it has been reported that huddling does not appreciably affect dose of toxicant received [14]. In between exposures the animals were housed in special accomodations near the inhalation unit whilst after the last exposure they were transferred to permanent accomodation.

*2. Smoke generation*

Thick washers of the smoke composition (Haley and Weller, Draycott, Derbyshire, UK) of mean weight 1.7 g were ignited on electrically heated nichrome wire, the concentrations being varied by igniting different numbers of washers. The resultant coloured smoke was mixed in a static 10 $m^3$ chamber with high velocity jets.

*3. Exposure*

The animals were exposed to the freshly generated coloured smoke 1 h per day for 5 days each week until they received 200 exposures or, in the case of the guinea-pig high dose group, 75 exposures. In each case, corresponding groups of each species were exposed together starting with exposure of the controls to air in the chamber and finishing with the high dose group. As the concentration fell, during exposure of each test group, fresh washers of the pyrotechnic composition were ignited, in order to maintain the concentration of smoke. During the 20–30 min interval between exposure of each dose group the chamber was scrubbed clean of excreta: at the end of exposure of the high dose group, the chamber was well washed twice.

During each exposure, sampling of the smoke was carried out by drawing air through fibre-glass filters for a known time. The filters were absolute with no bypass. Weighing of the impacted mass of smoke allowed the concentration in the chamber to be calculated. The intention was that the ratio of concentration and dose ($Ct$, concentration $\times$ time) should be 1:3:10. (Table 2). Particle size of the smoke was determined using an Anderson particle sizer. No interim kill was carried out, and all animals alive at the end of exposure were observed until they died or until the end of the 6 month observation period.

*4. Observations*

Animals were examined during the exposure period for ill-health of abnormal behaviour. They were weighed weekly during exposure and monthly during the ensuing observation period.

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* Due to death after supply less than 200 were used in the study.

*5. Sacrifice*

Animals showing signs of ill-health and all those remaining alive at the end of the study (17 months after the start of exposure) were killed with pentobarbitone sodium. After sacrifice the animals' bodies were weighed and the following organs removed and weighed: heart, lungs, both kidneys, liver, spleen, thymus and both adrenal glands. In addition the larynx, trachea, stomach, small and large intestines and pancreas were removed but not weighed. Cervical lymph nodes were taken only in the case of the rats and guinea-pigs. In all cases portions from all the foregoing organs as well as any observed at autopsy to be abnormal were processed histologically. In a few cases organs, mostly mouse thymuses, were not found at autopsy. After fixation in neutral buffered formalin, sections 3 $\mu$m thick were cut and stained with haematoxylin and eosin. At histological examination certain changes were graded visually. Histological data were recorded on a PLACES data acquisition system supplied by Apoloco Ltd, 90 King Street, Newcastle-under-Lyme, Staffs, England, ST5 IJB, UK, this package being run on a PDP 11/23 minicomputer (Digital Equipment Corporation, Maynard, Mass., USA).

*6. Statistics*

The frequency of histological changes in surviving animals from the test groups was compared with that in corresponding control groups using Fisher's exact test. Additionally a test for a trend in the frequency of histological changes against dose was performed [15]. Where histological changes were graded statistical tests were generally performed on all grades of severity of the lesion, or the severe form alone because tests on the milder form alone would have been without validity, the data not being independent. Numbers of decedents were insufficient for statistical treatment of the frequency of histological changes in these animals.

Weight gain in the animals was analysed in two ways. Mean weight of test groups divided by the mean weight of their corresponding control groups was regressed against time. Also during the observation period weight of test groups was compared with weight of control groups at various time intervals using Student's $t$-test.

Since the weights of many of the organs were not normally distributed, the non-parametric Kolmogorov–Smirnov test [16] which indicates significance, where the difference between two cumulative distributions exceeds a critical value, as well as Student's $t$-test were applied to the absolute organ weights, the organ weights relative to the whole body weights and to total body weights at sacrifice.

*7. Ames test*

Solvent Yellow 33 (Colour Index 47,000, Society of Dyers and Colourists, 1979 [4]) was obtained from ICI Dyestuffs Ltd, Blackley, Manchester and

L.B. Holliday Ltd, Deighton, Hudderfield, West Yorkshire. Disperse Orange 11 (Colour Index 60,700) was obtained from L.B. Holliday Ltd. Representative samples of each dye were dissolved in dimethylsulphoxide and independently tested in the Ames test through an incremental concentration range of 0.195 $\mu$g to 100 $\mu$g per plate both in the presence and absence of Aroclor-induced rat liver microsomes. Each sample was tested in triplicate, using three plates at each dye concentration, with Salmonella tryphimurium indicator strains TA 98, TA 100, TA 1537 and TA 1537 R+. Protocols were those recommended by Ames et al. [17]. Strains TA 98, 100 and 1537 were obtained from the Ames laboratory. Strain TA 1537 R+ was isolated after transfer of plasmid pKM 101 from Salmonella tryphimurium strain TA 100 to an intermediate host, Escherischia coli CM 611, followed by transfer to strain Salmonella TA 1537. The strain TA 1537 R+ has a naturally high spontaneous reversion rate to histidine independance but consistently shows greater sensitivity to anthraquinone dyes than does strain TA 1537 (B. Morris, unpublished data). The polychlorinated biphenyl Aroclor 1254 was a gift from Monsanto Ltd. Its usage in the induction of P450 enzymes was as described by Ames et al. [17].

## Results

### *1. Analysis*

The mean concentrations, daily and total doses achieved were, apart from the high dose group of guinea-pigs, close to what was desired (Table 2). The

TABLE 2

Mean daily concentration of smoke during exposure, mean daily dose (*Ct*, concentration × time) and total dose (*Ct*).

| Species | Dose group | Concentration* (mg $m^{-3}$) | No. of exposures | Daily dose (mg min $m^{-3}$) | Total dose (g min $m^{-3}$) |
|---|---|---|---|---|---|
| Mice | Low | 111 ± 9 | 200 | 6,660 | 1,332 |
| | Medium | 307 ± 20 | 200 | 18,420 | 3,684 |
| | High | 1005 ± 78 | 200 | 60,000 | 12,060 |
| Rats | Low | 111 ± 9 | 200 | 6,660 | 1,332 |
| | Medium | 307 ± 20 | 200 | 18,420 | 3,684 |
| | High | 1005 ± 78 | 200 | 60,300 | 12,060 |
| Guinea | Low | 111 ± 9 | 200 | 6,660 | 1,332 |
| Pigs | Medium | 307 ± 20 | 200 | 18,420 | 3,684 |
| | High | 1009 ± 105 | 75 | 60,540 | 4,540 |

*Mean ± SD of all the exposure concentrations throughout the experiment.
The total dose received by the high group of guinea-pigs was lower than that received by the other high dose groups because of withdrawal of guinea-pigs from exposure.

TABLE 3

Cumulative total of deaths by months during study

| Species | Dose group | Time | | | | | | | | | | (Including final kill) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Exposure | | | 11 | 12 | 13 | 14 | 15 | 16 | 17 | |
| | | 4 | 8 | 10 | | | | | | | | |
| Mice | Control | 2 | 3 | 5 | 8 | 9 | 10 | 12 | 15 | 16 | 22 | 100 |
| | Low | 1 | 2 | 3 | 5 | 5 | 5 | 8 | 10 | 15 | 17 | 100 |
| | Medium | 0 | 1 | 3 | 3 | 5 | 7 | 8 | 8 | 13 | 16 | 100 |
| | High | 2 | 8 | 12 | 14 | 15 | 16 | 19 | 19 | 23 | 25 | 100 |
| Rats | Control | 0 | 0 | 0 | 1 | 1 | 2 | 3 | 6 | 6 | 6 | 50 |
| | Low | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 7 | 50 |
| | Medium | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 3 | 6 | 6 | 50 |
| | High | 4 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 50 |
| Guinea Pigs | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 45 |
| | Low | 0 | 0 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 47 |
| | Medium | 2 | 4 | 5 | 6 | 6 | 6 | 6 | 6 | 7 | 8 | 48 |
| | High | 22* | 22 | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 46 |

*75 exposures only.

exception was caused by the withdrawal of the guinea-pigs from exposure because of the high mortality in that species.

The mass median diameter of the smoke was 0.94 $\mu$m, 1.55 $\mu$m and 1.10 $\mu$m for the low, median and high dose groups respectively.

*2. Decedents*

Study of the survival data (Table 3) shows that neither in mice nor rats were there great differences in the numbers of animals remaining to be sacrified at the end of the study. In the rats, deaths in the high dose group appeared to occur earlier than in the other groups and there was a significant increase in total mortality in this group compared to the controls over the total exposure period ($p=0.013$). In the guinea-pigs a large number of deaths occurred within a very short period in the 4th month of the study, for which reason the high dose guinea-pigs were not further exposed.

Decedent mice were found to have a variety of histological changes in the lungs including peribronchial and perivascular lymphocytic infiltration, excess macrophages, alveolitis and pneumonitis. Alveolar oedema was seen in one case. These changes were more frequent in the test groups than the controls. Rats which died before the end of the study showed fewer pulmonary changes but macrophages were particularly frequent in the middle dose group. Decedent guinea-pigs frequently showed mild alveolitis. This occurred in 17/23 high

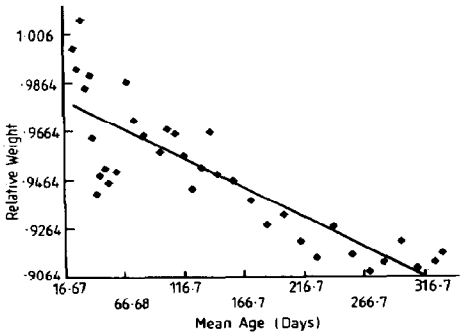


Fig. 1. Weight of low dose group of mice relative to control group during exposure period.

dose group of animals practically all of which died during exposure; it was also present in the other two guinea-pigs dying during exposure, both of these animals coming from the middle dose group. By contrast other decedent guinea-pigs, which died during the observation period, had little evidence of alveolitis. Tumours were not frequently found in the decedents in any species, most particular tumour types being present in single instances only. An exception was alveologenic carcinoma in mouse lungs. This was found in 5 out of 22 control mouse decedents and less frequently in the dosed groups.

*3. Growth*

Statistically significant differences between the weight of test groups and corresponding controls were seen at the end of the exposure period in all species. Moreover when relative weight was regressed against time this variable was seen to fall throughout the exposure period. An example is given in Fig. 1 for the low dose group of mice. Similar falls were seen in the higher dose groups although these decreases in relative weights were greater so that the effect appeared to be dose related.

After cessation of exposure the animals tended to regain their lost weight so that in the rats, the only statistically significant difference in group mean weight which remained at death, was that between the high dose group and controls (375 g against 397 g, $p=0.025$). This difference was also significant using the non-parametric test ($p=0.045$). Similar phenomena were observed in mice except that at sacrifice it was the high group that was statistically and significantly lighter than all the others ($p=0.05$ vs medium, low and control). Using the non-parametric test the difference between high dose and control groups was statistically significant. ($p<0.05$)

In the guinea-pigs the effects of exposure on the high group can be seen by comparing the mean weight at just before cessation of exposure in that group ($625\pm92$ g, $\bar{x}\pm$S.D., $n=45$) with the controls ($814\pm93$ g, $\bar{x}\pm$S.D., $n=45$). Similar though smaller differences were seen with the intermediate groups and

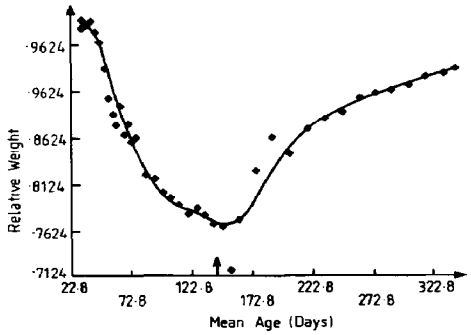


Fig. 2. Weight of high dose group of guinea-pigs relative to control group during and after exposure.

using Student's $t$-test all these differences were significant statistically. At 12 months when all dosing ceased the medium dose group was statistically and significantly lighter than the controls ($p=0.002$). By contrast the high dose group where exposure had ceased months earlier showed recovery and had now overtaken the medium dose group (Fig. 2). At sacrifice. when a further 5 months had elapsed, no meaningful statistical differences were observed between the guinea-pig test groups including controls.

*4. Organ weights*

These data have not been presented in detail for the sake of brevity. In mice many of the organs showed intergroup differences, either when examined in absolute terms or relative to body weight. The relative lung weights exhibited a trend of increasing with dose. Using the non-parametric test the distribution of high dose lungs contained a significant ($p<0.01$) proportion of lungs heavier than expected. Reductions in kidney and liver weights in mice were commensurate with a reduction in body weights so that relative weights of those organs did not differ within groups. It was noteworthy that the weight of the heart was very stable (i.e. did not fall with body mass). A very marked reduction in thymus weight with dose ($p<0.02$) was not entirely accounted for by total body weight reduction: thus the high dose group thymus data showed a statistically significant reduction ($p=0.03$) compared to the other groups. Mouse spleens in all three test groups were significantly lighter than controls ($p<0.03$). This significance became marginal when relative organ weights were considered.

Amongst the rats neither absolute nor relative weights of lungs, heart, thymus and kidney showed significant differences between corresponding groups. Differences in absolute hepatic and splenic weights were removed after adjustment for body weight. Conversely such adjustment in adrenal weight revealed significant but almost uninterpretable differences. In order to aid interpretation the weights of the right and left adrenals were added together.

TABLE 4

Selected pathology in mouse that survived to the end of the story

| Organ/Lesion | Dose Group | | | |
|---|---|---|---|---|
| | Control | Low | Medium | High |
| Total Lungs | (78)[a] | (83) | (84) | (75) |
| Peribronchial lymphocytic infiltration | 49 | 46 | 49 | 43 |
| Perivascular lymphocytic infiltration | 28 | 31 | 32 | 28 |
| Macrophage infiltration | 0 | 1 | 2 | 28[c] |
| Alveologenic carcinoma | 9 | 13 | 7 | 5 |
| Total larynx | (78) | (82)[d] | (83)[d] | (75) |
| Lymphocytic infiltration | 32 | 43 | 44 | 46[b] |
| Dilated mucous glands | 0 | 1 | 0 | 1 |
| Total trachea | (78) | (83) | (84) | (75) |
| Lymphocytic infiltration | 23 | 30 | 23 | 30 |
| Dilated mucous glands | 0 | 4 | 6[b] | 6[b] |
| *Extrarespiratory organs* | | | | |
| Total number of animals with tumours | 12 | 14 | 10 | 7 |
| Benign tumours | 2 | 2 | 1 | 1 |
| Malignant tumours | 10 | 13 | 9 | 6 |

[a]Figure in brackets is number of organs examined.
[b]$p < 0.05$ compared with controls by Fisher's exact test.
[c]$p < 0.01$ compared with controls by Fisher's exact test.
[d]One damaged during processing.

In this case the weight of the medium dose adrenals were significantly larger than the other groups and when body weight was taken into account the weights of both medium and high dose adrenals were greater than that of control or low dose group glands.

With the guinea-pigs the high dose group of animals was considered separately (see below). In the others, no significant differences were found in absolute or relative weights of lungs, thymus, spleen or adrenals. Where absolute data showed significant reductions (e.g. liver: medium vs controls $p=0.01$), whole body weight adjustment reduced the level of significance to $p=0.02$. In the case of the heart statistically significant reductions in weight were abolished by consideration of the whole body weight, and the same was true of kidneys. Interpretation of organ weights in the 24 high dose group guinea-pig survivors is difficult. As far as total body weight, lungs, liver and adrenals, the weights in this group were greater than the controls or remaining test groups. For thymus, spleen and kidneys the weights of the high dose group organs fell between control and low dose groups whilst for the heart it lay between low and medium dose groups.

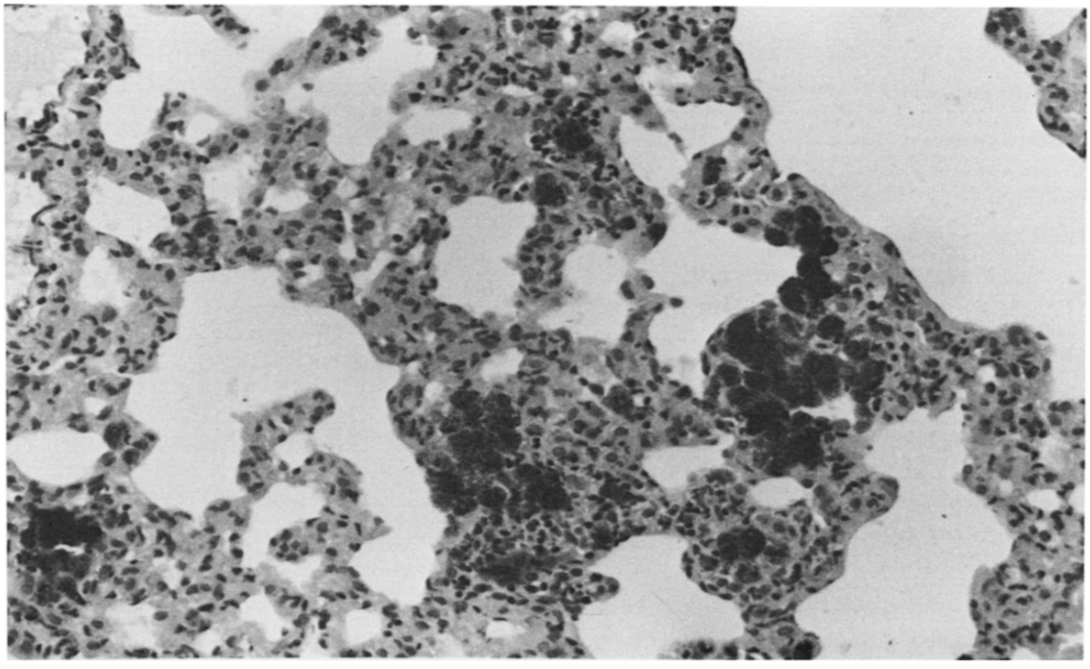

Fig. 3. Lung of rat from high dose group showing macrophages containing black granules. H & E ×220.

TABLE 5

Selected pathology in rats that survived to the end of the study

| Organ/Lesion | Dose group | | | |
|---|---|---|---|---|
| | Control | Low | Medium | High |
| *Lungs* | (44)[a] | (43) | (44) | (44) |
| Peribronchial lymphocytic infiltration | 42 | 42 | 41 | 44 |
| Perivascular lymphocytic infiltration | 19 | 18 | 32[c] | 25 |
| Macrophages with granules | 0 | 3 | 37[c] | 42[c] |
| Lymphocytic foci | 0 | 0 | 4 | 6 |
| Macrophages (non-granular) | 0 | 1 | 2 | 1 |
| *Larynx* | (44) | (43) | (44) | (44) |
| Submucosal lymphocytes | 12 | 13 | 13 | 7 |
| Dilated mucous glands | 9 | 16 | 17 | 9 |
| *Trachea* | (44) | (43) | (44) | (44) |
| Submucosal lymphocytes | 23 | 14 | 13 | 12[b] |
| Dilated mucous glands | 0 | 7[c] | 9[c] | 11[c] |
| Total number of animals with tumours | (44) | (43) | (44) | (44) |
| | 2 | 4 | 5 | 6 |

[a]Figures in parentheses are number of organs examined.
[b]$p < 0.05$ compared to controls by Fisher's test.
[c]$p < 0.01$ compared to controls by Fisher's test.

TABLE 6

Selected pathology in guinea pigs that survived to the end of the study

| Organ/Lesion | Dose group | | | |
|---|---|---|---|---|
| | Control | Low | Medium | High |
| *Lungs* | (42)[a] | (44) | (40) | (24) |
| Peribronchial lymphocytic infiltration | 13 | 34[c] | 20 | 15[b] |
| Perivascular lymphocytic infiltration + | 2 | 0 | 0 | 0 |
| Perivascular lymphocytic infiltration (all) | 18 | 3[c] | 0[b] | 0[b] |
| Macrophages + | 0 | 0 | 1 | 0 |
| Macrophages (all) | 0 | 2 | 6[b] | 3[b] |
| Adenoma | 1 | 1 | 3 | 0 |
| *Larynx* | | | | |
| Submucosal lymphocytes + | 3 | 10 | 13[c] | 11[c] |
| Submucosal lymphocytes (all) | 3 | 13[b] | 14[c] | 13[c] |
| *Trachea* | | | | |
| Submucosal lymphocytes + | 2 | 0 | 0 | 0 |
| Submucosal lymphocytes (all) | 19 | 20 | 28[b] | 15 |

[a]Figures in parentheses are number of organs examined.
[b]$p < 0.05$ compared with controls by Fisher's test.
[c]$p < 0.01$ compared with controls by Fisher's test.

*5. Histological findings in survivors*

Mice showed a large variety of histological changes in the lungs. Some were graded at examination and the prevalence of both the severe form ( + ) and all forms together are given in some cases. The more noteworthy changes are presented in Table 4. Macrophage infiltration was significantly more frequent in the high dose group than the controls ($p < 0.01$) whilst peribronchial lymphocytic infiltration was seen in over half of all groups. Perivascular lymphocyte infiltration was somewhat less frequent but still often observed and likewise not dose related. Alveologenic carcinoma was present in mice from all dose groups but most frequent in the low dose group. The larynx showed a high frequency of lymphocytic infiltration and this change was significantly more frequent in the high dose group than the controls ($p < 0.05$). In other respects, including the ciliated epithelium, the larynx appeared normal: there was no evidence of metaplasia. Similar changes were present in the trachea; however, there was no statistically significant difference between test groups and controls in the prevalence of lymphocytic infiltration. In that organ the frequency of dilated mucous glands was significantly greater in both high and medium dose groups than controls ($p < 0.05$, both cases), whilst the same change in the larynx was unrelated to dose. Other than alveologenic carcinoma, tumours were infrequently observed, particular types being present only in single instances.

TABLE 7

Reversion of Salmonella tryphmurium strain TA1537R+ by Solvent Yellow 33 (ICI and Holliday) A and Disperse Orange 11 B in the presence or absence of Aroclor 1254 induced rat liver homogenate (S9+or−).

| Source | Dye conc. ($g \cdot plate^{-1}$) | A: Number of reversions[1] induced by Solvent Yellow 33 | | | | | |
|---|---|---|---|---|---|---|---|
| | | Test 1 | | Test 2 | | Test 3 | |
| | | (+) | (−) | (+) | (−) | (+) | (−) |
| ICI | 100 | 1041 | 970 | 1056 | 1166 | 988 | 897 |
| Dyestuffs | 50 | 840 | 898 | 363 | 1062 | 884 | 676 |
| | 25 | 954 | 984 | 712 | 1136 | 1144 | 860 |
| | 12.5 | 940 | 862 | 856 | 972 | 1268 | 656 |
| | 6.25 | 890 | 788 | 777 | 1066 | 760 | 672 |
| | 3.125 | 584 | 706 | 382 | 820 | 436 | 692 |
| | 1.56 | 463 | 642 | 232 | 644 | 620 | 632 |
| | 0.78 | 432 | 628 | 348 | 620 | 464 | 712 |
| | 0.39 | 426 | 650 | 144 | 598 | 512 | 508 |
| | 0.195 | 403 | 528 | 160 | 610 | 655 | 422 |
| | 0 | 397 | 384 | 145 | 564 | 591 | 430 |
| L.B. | 100 | 660 | 926 | 632 | 812 | 463 | 1140 |
| Holliday | 50 | 619 | 959 | 684 | 872 | 520 | 1122 |
| | 25 | 630 | 923 | 856 | 756 | 562 | 1004 |
| | 12.5 | 541 | 877 | 804 | 788 | 336 | 1136 |
| | 6.25 | 412 | 743 | 556 | 756 | 266 | 760 |
| | 3.125 | 292 | 741 | 422 | 620 | 255 | 840 |
| | 1.56 | 267 | 585 | 314 | 424 | 214 | 820 |
| | 0.78 | 262 | 498 | 348 | 338 | 203 | 536 |
| | 0.39 | 225 | 428 | 276 | 296 | 189 | 566 |
| | 0.195 | 298 | 314 | 252 | 312 | 143 | 434 |
| | 0 | 199 | 244 | 270 | 272 | 154 | 448 |
| | | B: Number of revertants[1] induced by Disperse Orange 11 | | | | | |
| L.B. | 100 | 1940 | 3024 | 2324 | 2052 | 2500 | 1864 |
| Holliday | 50 | 1756 | 2565 | 2196 | 2168 | 2689 | 1728 |
| | 25 | 1732 | 2668 | 2300 | 2160 | 1676 | 656 |
| | 12.5 | 1100 | 2520 | 2084 | 2140 | 1260 | 484 |
| | 6.25 | 1012 | 2460 | 1340 | 1424 | 948 | 754 |
| | 3.125 | 680 | 1524 | 1092 | 1148 | 494 | 632 |
| | 1.56 | 450 | 1204 | 630 | 1020 | 432 | 511 |
| | 0.78 | 408 | 1010 | 452 | 850 | 304 | 428 |
| | 0.39 | 288 | 522 | 392 | 746 | 232 | 250 |
| | 0.195 | 288 | 468 | 402 | 488 | 326 | 184 |
| | 0 | 307 | 444 | 379 | 507 | 331 | 257 |

[1]The numbers of revertants tabulated at each level of dye concentration represents an average calculated from the total numbers of revertants enumerated from triplicate plates.

No dose-related increase in tumours grouped regardless of histological type, was seen in the mice (Table 4). Rats also showed several histological changes in the lung. In the high and medium dose groups there was a higher frequency of macrophage infiltration (Fig. 3, Table 5). The difference was statistically significant ($p<0.01$, both groups). These macrophages often exhibited many small dark granules which, neither contained dye nor stained for iron using Perl's method. In the trachea but not the larynx dilated mucous glands were more frequently seen ($p<0.01$, all cases) in the three test groups than in the controls: lymphocytic infiltrates, while seen in those organs, were not dose-related. Whilst no individual tumour was statistically and significantly more frequent in any test group than in the controls there is some indication of a trend in all tumours taken together.

Histological changes were frequent in the lungs of the guinea-pigs, a particularly susceptible species in which the high dose group had to be withdrawn from treatment. This group showed statistically significant increases in the frequency of two pulmonary histological changes namely the presence of excess macrophages and peribronchial lymphocytic infiltration (Table 6). Perivascular lymphocytic infiltration seemed to be less common in test groups than controls. In the larynx submucosal lymphocytic infiltration was usually severe and significantly more prevalent in both high and medium groups than controls ($p<0.01$). In the trachea, the same change, regardless of severity was statistically significantly more frequent in the medium dose group but not the highest, when compared to the controls. It will be recollected that in this species the medium dose group was the highest dose to receive the full protocol of exposure. Tumours were not often seen in the guinea-pigs, except for ovarian cystadenomata: only one was classified as malignant, a poorly differentiated fibrosarcoma of the uterus in the control group.

In all three species a number of findings were observed that are commonly recognised histological changes in that species. Noteworthy amongt these were nephropathy in rats (Table 5) and lymphocytic foci in guinea-pigs (Table 6).

*6. Ames test*

Solvent Yellow 33 and Disperge Orange 11 were both mutagenic for the indicator strain TA 1537 R+ whether in the presence or not of S9-mix (Table 7). Although samples of Solvent Yellow 33 (ICI Dyestuffs Ltd and L.B. Holliday Ltd) were mutagenic that from ICI was only marginally so. The sample from L.B. Holliday Ltd was mutagenic at concentrations of dye at or above 6.25 $\mu$g per plate in each of the three tests.

Disperse Orange 11 was the more mutagenic of the dyes tested. There was an increase in the numbers of revertants per plate of greater than six times the number observed in the solvent plates. The mutagenic response was evident at dye concentrations above 3.125 $\mu$g per plate.

The numbers of revertants recovered from strain TA 1537 R+ was generally

high on plates containing dyes or solvent alone and is a property likely to increase mutagen sensitivity in this strain. Of the samples tested none was mutagenic for strains TA 98, TA 100 or TA 1537 irrespective of the presence of S9 or its absence.

The number of revertant colonies seen for each strain on plates containing known mutagens were within the ranges expected.

**Discussion**

The large number of deaths observed in the high dose group of guinea-pigs was not unprecedented since a similar phenomenon was observed in previous inhalation studies using that species [1,18]. It is doubtless a reflection of the peculiar sensitivity of guinea-pigs to the stress of inhalation of particulates [19]. Although these animals usually exhibited some degree of alveolitis it is likely, on the basis of previous studies of the known sensitivity of guinea pigs, that they died of bronchospasm. The other two species, whose survival was not greatly affected by exposure (mice and rats) exhibited a variety of histological changes but it was often not possible to ascribe a cause of death. In no case was there evidence of dye retention in the lungs.

Although only the guinea-pigs showed diminished survival, the smoke clearly was toxic to all test groups of the three species since their relative weights dropped during exposure. Organ specific toxicity however seems to have been more or less confined to the respiratory tract, most histological changes observed in other organs being recognised background pathology of the species. Such changes included nephropathy in rats which is generally considered a degenerative disease [20]. Lymphocytic foci (lymphoid nodules) are spontaneous frequently-observed lesions in guinea-pigs, whose aetiology is still uncertain [21,22].

The increase in relative lung weight-loss, observed in the test groups of mice, was not accompanied by any histological change, such as oedema, which would easily account for its presence. It seems more likely that it resulted from the dose-related decrease in weight-gain seen in this species, with sparing of lung mass. The statistically significant reduction in thymus weight was not almost certainly a stress effect, which made location of that organ sometimes difficult.

In the surviving animals a very noteworthy and significant difference from the previously tested coloured smoke was the absence of retained dye in the lungs of any species and the absence of sheets of packed macrophages as was previously seen in rats. In the study of Marrs at al. [1] both dye retention and the massive macrophage response were believed to be effects of Solvent Green 3, a dye which is not contained in the present smoke. Thus, since both smokes contained Solvent Yellow 33, it seems that Disperse Orange 11 is rapidly cleared like the yellow dye and Disperse Red 9. Therefore the various findings in the respiratory tracts of the animals represent the less specific aspects of smoke

toxicity which are probably common to all military smokes and were caused by the non-dye constituents. These included lymphocytic infiltration in the larynx and trachea in the mice and guinea-pigs, unaccompanied by changes in other structures such as the goblet cells and ciliated epithelium. Likewise, the dilated mucous glands seen in the trachea in the mice and rats, were almost certainly unrelated to the dyes in the smoke. The relatively unspectacular nature of the findings is explicable on the basis of previous studies of the products of reaction between potassium chlorate and lactose. The main components of the resulting smoke are carbon dioxide, water and potassium chloride [23]. Where incomplete combustion occurs carbon monoxide will be present, but in no circumstances are products found, which are likely to produce acute lung damage.

In all three species, effects attributable to the dyes were conspicuous by their absence. Even the macrophages containing small dark granules, observed in the test group rats only, neither contained dye nor stained for iron. These granule-containing macrophages have been observed in other smoke studies here. The most likely explanation for the granules is that they are carbon, derived from incomplete pyrolysis of the lactose present in the pyrotechnic mixture. It is further, reassuring, that severe toxic effects on the respiratory tracts as observed with certain military white smokes, were not seen in the present study [24–26].

Neither the pattern of exposure nor the duration of the study allow this experiment to be described, in any sense, as a carcinogenicity test. Some tumours were seen, however, but the only one of any great frequency was the mouse alveologenic carcinoma. In no case was a particular tumour type present statistically and significantly more often in a test group than a control group. However the constituent dyes of the smoke were mutagenic and a previous study [11] has shown one of them to be a carcinogen. Moreover the Ames test result reported in the present study has shown both dyes to be mutagenic to one strain of Salmonella, marginally so in the case of Solvent Yellow 33. This is not the first indication that Solvent Yellow 33 may be mutagenic since Moore et al. [27] found 2-(2-quinolyl)-1,3-indanedione to be positive in the Ames test with indicator strain TA 1537. It was also positive with strains TA 102 and 104 with and without S9, and weakly positive with S9 using Salmonella indicator strain TA 100. Moreover the material was mutagenic in the L5178Y mouse lymphoma cell thymidine kinase assay, but did not induce sister chromatid exchange.

## Conclusions

The present study has shown that this coloured smoke is, when administered repeatedly to small laboratory animals, remarkably lacking in organ specific toxicity outside the respiratory tract. However, because both dyes are muta-

genic in the Ames test and one an animal carcinogen [10,11] their genotoxic potential must give rise to concern. To elucidate this aspect of the toxicity of the smoke, a longer term study would be required.

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