Tumor Initiatory Activity of Some Chloromononitrobenzenes and Other Compounds

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Summary

Treatment of mouse skin with pentachloronitrobenzene and each of the 3 tetrachloronitrobenzenes resulted in multiple papilloma formation during subsequent treatment with croton oil. The highest yield of papillomas was obtained with 2,3,4,5-tetrachloronitrobenzene, the only compound of the 4 that has a stable nitro group and does not give rise to appreciable mercapturic acid in the rabbit. It is suggested that the tumor initiatory activity observed is due to hydroxylamine derivatives formed as intermediates in metabolic reduction of the nitro groups. Chloroacetone and β-bromopropionic acid, but not N-ethylmaleimide, also acted as tumor initiators. The chloronitrobenzenes did not affect skin carcinogenesis in the mouse by benzo[a]pyrene.

Introduction

Earlier experiments in this laboratory have shown that 4-nitroquinoline N-oxide, already known to be a mutagen (16) and carcinogen (14), also markedly inhibits the carcinogenic activity of benzo[a]pyrene when applied throughout the course of carcinogenesis, either alternately with the BP (2) or in the same solution (20). Single doses of NQO also inhibit the tumor initiatory activity of a single dose of BP (19).

The most striking chemical reactivity of NQO at physiologic pH is with compounds possessing the thiol (—SH) group, including the important tissue constituents cysteine and glutathione (12). In this reaction the unusually reactive nitro group is eliminated as nitrite, the thiol sulfur becoming bound to the quinoline ring in its place. Nitro groups that are labile, though to a much lesser extent, are also found in some of the higher chlorinated nitrobenzenes (4), and the experiments described in the present paper were carried out to see whether 4 of these compounds, pentachloronitrobenzene and the isomeric tetrachloronitrobenzenes, might also possess some degree of carcinogenicity or of inhibitory activity against BP carcinogenesis.

Apart from the possibility of correlating any such activity with the known behavior of these substances on metabolism, it is important to know whether those substances with actual or potential uses in agriculture or industry present any carcinogenic hazard. Of the above compounds, 2,3,5,6-TCNB has been found useful in inhibiting dry rot and sprouting of seed potatoes, and Brook (5) has shown that the 2,3,4,5- and 2,3,4,6-isomers also possess fungicidal activity, as do a variety of aromatic compounds of related structure, such as the dichlorodinitrobenzoates recently tested by Summers and Turner (22). It is relevant that fungicidal activity was demonstrated in NQO (15) and β-propionic lactone (2) before they were recognized as carcinogens.

The experiments reported here have not, in fact, demonstrated any appreciable activity as carcinogens or inhibitors of carcinogenesis; however, it has been found that all 4 compounds are initiators of skin tumor formation when croton oil is employed as a promoting agent. For various reasons, chloroacetone, N-ethylmaleimide, and β-bromopropionic acid were included in these tests; of these, chloroacetone and β-BPA were also found to initiate development of skin tumors.

Materials and Methods

PCNB. Technical PCNB (Plant Protection, Ltd.) was twice recrystallized from boiling ethanol and obtained as fine leaflets, m.p. 145°-146°C.

2,3,4,5-TCNB. 1,2,3,4-Tetrachlorobenzene (Plant Protection, Ltd.) was nitrated with fuming nitric acid (sp. gr. 1.5) for 15 min under gentle reflux. 2,3,4,5-TCNB was obtained in high yield as a slightly creamy crystalline solid, m.p. 65°-66°C after crystallization from ethanol.

2,3,4,6-TCNB. 1,2,3,5-Tetrachlorobenzene, prepared from 2,4,6-trichloroaniline by the Sandmeyer reaction, was nitrated with fuming nitric acid to give 2,3,4,6-TCNB, m.p. 40°-41°C after crystallization from aqueous ethanol.

2,3,5,6-TCNB. Technical 2,3,5,6-TCNB (Tecnazene, 1,2,4,5-tetrachloro-3-nitrobenzene; Plant Protection, Ltd.) was dissolved in hot ethanol, filtered, and allowed to crystallize. It was obtained as colorless rhombs, m.p. 99°-100°C.

OTHER COMPOUNDS. NEM and β-BPA, both from British Drug Houses, were used without further purification. MgO-stabilized chloroacetone, from the same source, was filtered and distilled at 118°C before use. BP, croton oil, and acetone were as used in the previous communication (19).

ANIMALS. The mice were the outbred stock albino usually employed in these laboratories for carcinogenicity tests. They were housed in galvanized boxes containing 5 animals, and were fed Thomson No. 1 cube diet and tap water. Mice were 1st treated at 6-8 weeks of age, except in 1 inhibition experiment treated at 6-8 weeks of age, except in 1 inhibition experiment when they were 4-5 weeks old. Hair was removed from their
backs with electric clippers at the start of each experiment, and at intervals of a few weeks thereafter as necessary.

**Preliminary Carcinogenicity Tests.** These were carried out with 2,3,4,6- and 2,3,5,6-TCNB only, with the use of groups of 15 male and 15 female mice for each compound. Each mouse received 0.2 ml of 0.3% acetone solution twice weekly. After 14 weeks, 5 male and 5 female mice on each compound were changed to twice weekly applications of croton oil (0.2 ml, 0.5% in acetone). As many papillomas arose on the skin of these croton oil-treated mice, the following experiment was carried out.

**Tests for Tumor Initiatory Activity.** The compounds examined were PCNB, 2,3,4,5-, 2,3,4,6-, and 2,3,5,6-TCNB, chloroacetone, NEM, and β-BPA. All were 0.3% in acetone except β-BPA, which, owing to its inactivity in earlier carcinogenicity tests (18), was applied in 2.5% solution.

Applications of 0.2 ml were made twice weekly for 12 weeks to the clipped backs of the mice. Ten males and 10 females were used for each compound and for an acetone-treated control group. All the mice were then treated with croton oil for 20 weeks, and surviving mice were killed after another 20 weeks without treatment.

The numbers of mice bearing macroscopic tumors and total numbers of tumors were determined weekly during croton oil treatment and then fortnightly; papillomas less than 1 mm in diameter and not persisting for at least 3 weeks were ignored. The figures obtained in the various groups at 10, 20, 30, and 40 weeks from the start of croton oil treatment are given separately for males and females in Table 1. As in the previous paper (19), the figures are noncumulative and show the expected occurrence of some regressions toward the end of the experiment. They include, however, tumors present at death on mice already dead. These are excluded by calculating the average numbers of tumors/surviving mouse, which are plotted against the duration of croton oil treatment in Charts 1 (chloronitrobenzenes) and 2 (chloroacetone, NEM, and β-BPA). These averages are liable to show occasional sudden drops on the death of a mouse with an unusually large number of papillomas, but are thought to give a fairer representation of the differences between the various groups of mice under treatment.

A number of the larger skin tumors present at death during and at the end of the experiment were sectioned and stained for histologic examination.

**Tests for Inhibition of BP Carcinogenesis of Mouse Skin.** Two series of tests were carried out. In 1 series the chloronitrobenzenes were applied (0.2 ml/mouse, 0.3% in acetone) each Tuesday and the BP (0.2 ml, 0.2% in acetone) each Friday. In the 2nd series the chloronitrobenzenes and BP were applied weekly in 0.2 ml of acetone containing the above concentrations of each compound. Spectrophotometric examinations of the mixed solutions showed that no detectable interaction occurred when they were stored for a few months at 4°C.

Each test group contained 15 male and 15 female mice, as did a control group treated with BP alone in each experiment. When papillomas began to appear, weekly counts were made of the numbers of tumor-bearing mice and of the total numbers of tumors in each group.

### Results

**Carcinogenicity Tests on 2,3,4,6- and 2,3,5,6-TCNB.** Of the 20 mice treated throughout with 2,3,4,6-TCNB alone, 5 survived to over 60 weeks, but no skin lesions attributable to the treatment were seen. Four males and 1 female out of 20 on 2,3,5,6-TCNB survived to 90 weeks. This female had the only skin lesion in the group, a very small sebaceous adenoma, which was not thought to be associated with the treatment.

### Table 1

**Incidence of Skin Tumors on Mice during and after Treatment with Croton Oil, Following Applications of Some Chloromononitrobenzenes and Other Compounds**

| Test Compound                  | Survivors at 10 wk | Survivors at 20 wk | Survivors at 30 wk | Survivors at 40 wk | Mice with Tumors at 10 wk | Mice with Tumors at 20 wk | Mice with Tumors at 30 wk | Mice with Tumors at 40 wk | Total Tumors at 10 wk | Total Tumors at 20 wk | Total Tumors at 30 wk | Total Tumors at 40 wk | % Tumor Incidence |
|-------------------------------|--------------------|--------------------|--------------------|--------------------|-------------------------|--------------------------|--------------------------|--------------------------|------------------------|------------------------|------------------------|------------------------|----------------------|---------------------|
| Acetone controls              | M 10               | F 10               | M 10               | F 10               | M 10                    | F 10                      | M 10                     | F 10                      | 1                       | 1                      | 1                      | 1                      | 19                   |
| Pentachloronitrobenzene       | M 10               | F 10               | M 10               | F 10               | M 10                    | F 10                      | M 10                     | F 10                      | 1                       | 1                      | 1                      | 1                      | 19.5                 |
| 2,3,4,5-Tetrachloronitrobenzene | M 8               | F 8                | M 8               | F 8                | M 8                     | F 8                       | M 8                      | F 8                       | 1                       | 1                      | 1                      | 1                      | 20                   |
| 2,3,4,6-Tetrachloronitrobenzene | M 8               | F 8                | M 8               | F 8                | M 8                     | F 8                       | M 8                      | F 8                       | 1                       | 1                      | 1                      | 1                      | 20                   |
| 2,3,5,6-Tetrachloronitrobenzene | M 8               | F 8                | M 8               | F 8                | M 8                     | F 8                       | M 8                      | F 8                       | 1                       | 1                      | 1                      | 1                      | 20                   |
| Chloroacetone                 | M 9               | F 10               | M 9               | F 10               | M 9                     | F 10                      | M 9                      | F 10                      | 1                       | 1                      | 1                      | 1                      | 19.5                |
| N-Ethylmaleimide              | M 10              | F 10               | M 10              | F 10               | M 10                    | F 10                      | M 10                     | F 10                      | 1                       | 1                      | 1                      | 1                      | 20                   |
| β-Bromopropionic acid         | M 10              | F 10               | M 10              | F 10               | M 10                    | F 10                      | M 10                     | F 10                      | 1                       | 1                      | 1                      | 1                      | 20                   |

* Time is measured from start of croton oil treatment.

**END OF croton oil treatment.**
TUMOR INITIATORY ACTIVITY OF PCNB, 2,3,4,5-, 2,3,4,6-, and 2,3,5,6-TCNB. In the preliminary small-scale experiment, skin papillomas began to appear on mice treated with 2,3,4,6- and 2,3,5,6-TCNB after croton oil was applied for 12 and 8 weeks respectively, and over half the animals subsequently developed papillomas. The last mice treated with 2,3,4,6-TCNB and croton oil had to be killed 38 weeks after the 1st croton oil applications, and those in the 2,3,5,6-TCNB group had to be killed at 56 weeks. With the 2 exceptions mentioned below, the skin lesions induced were not malignant.

In the 2nd experiment, which was of larger scale (Table 1, Chart 1), it was found that treatment with each of the 4 chloronitrobenzenes tested caused multiple papilloma formation during subsequent applications of croton oil. All experimental groups began to show skin papillomas after 5-8 weeks of croton oil treatment. They became more numerous until 5-10 weeks after cessation of treatment, when some papillomas regressed.

When the total papillomas on male and female mice are considered together, the effects of PCNB and 2,3,4,6-TCNB appear very similar, that of 2,3,5,6-TCNB being a little but not markedly greater. (The sharp rise at 14-15 weeks in the plot of this group is due to the death of 4 mice from causes unrelated to the treatment.)

A larger total of papillomas was obtained on mice treated with 2,3,4,5-TCNB, despite the early loss of 4 mice in this group, and the average number of papillomas/survivor (Chart 1) was somewhat greater at all time intervals. (The hump at 15-18 weeks is largely due to 1 mouse with 18 papillomas, which was killed at 18 weeks owing to its poor condition.) Of the mice “at risk,” 50% treated with 2,3,4,5-TCNB or 2,3,5,6-TCNB before croton oil had skin papillomas after only 9 weeks of croton oil treatment, compared with 21 weeks in the acetone-treated control group.

TUMOR INITIATORY ACTIVITY OF CHLOROACETONE, NEM AND β-BPA. Treatment of mouse skin with 0.3% chloroacetone and 2.5% β-BPA also resulted in many more papillomas during subsequent croton oil treatment than occurred in the control animals (Table 1, Chart 2). As found with the chloronitrobenzenes, the papillomas began to appear some 5 weeks after the 1st croton oil applications, but a larger proportion regressed after cessation of treatment. This feature shows more clearly on the chart than in the table, owing to the exclusion from the chart of papillomas on mice already dead.

The only compound regarded as inactive at the dosage employed here is NEM. Mice treated with this compound developed only 50% more papillomas than did the controls and bore a maximum of 1.5 tumors/surviving mouse (controls, 0.75) at 30 weeks.

SEX DIFFERENCES IN THE INCIDENCE OF PAPILLOMAS. It can be seen from Table 1 that there were marked differences in the numbers of papillomas arising on male and on female mice. In most groups there was a tendency for male mice to develop papillomas earlier and in greater numbers than the females, but the reverse was true for the group treated with PCNB before croton oil. Thus, if only female mice are considered, PCNB was the most effective tumor initiator studied, though in the
male mice it was less effective than the 3 TCNB’s. The loss of some female mice with breast tumors cannot account for the sex difference observed, the significance of which is difficult to assess with the numbers of mice used here.

**STATISTICAL EVALUATION OF RESULTS.** As in the previous communication (19), the tumors in the various groups were assessed by analysis of variance at 10, 20, 30, and 40 weeks from the start of treatment with croton oil. The variance in response in the present experiments was markedly greater than in the experiments with BP used as tumor initiator (19).

The differences between the various test and control groups were not significant at 10 weeks. The effects of all 4 chloronitrobenzenes and of β-BPA were significant at 20 and 40 weeks, when total tumors were assessed, and at 20, 30, and 40 weeks, when assessment was made on the basis of tumors/survivor. The differences between 2,3,4,5-TCXB and the other chloronitrobenzenes were not significant at these times.

As expected, the small effects of NEM were not significant. More surprisingly, this was also true of the effects of chloroacetone. Whereas it is thought to be likely that the effects of chloroacetone would be significant in the middle range of this experiment if a more sensitive statistical analysis were performed, further animal tests with higher dosages used would seem desirable to substantiate the initiatory activity of this compound.

**OCCURRENCE OF MALIGNANT LESIONS IN INITIATION EXPERIMENTS.** Most of the tumors arising during croton oil treatment were small papillomas and were not examined histologically. Examination of tumors over 5 mm in diameter at the end of the experiment (earlier in a few cases) showed the presence of some malignant tumors, particularly in mice treated with β-BPA.

At the end of the main experiment, a single squamous-celled carcinoma, infiltrating to muscle, was present on the skin of a PCNB-treated mouse, and a basal-celled carcinoma was present on 1 mouse treated with 2,3,5,6-TCXB. An infiltrating squamous-celled carcinoma was also present on an acetone-treated control mouse killed only 31 weeks from the start of croton oil treatment. In the β-BPA group there was a squamous-celled carcinoma, penetrating the muscle, on 1 mouse at 33 weeks and on 3 more animals at the end of the experiment. One of these also had 3 keratoacanthomas. In addition, a mouse killed at 35 weeks had extensive lymphosarcoma.

No malignant tumors were found in mice treated with 2,3,4,5-TCNB, 2,3,4,6-TCNB, chloroacetone, or NEM, although a few benign skin lesions reached a diameter of 10 mm. However, in the preliminary experiment, 1 of the 10 mice treated with 2,3,4,6-TCNB had lymphosarcoma extensively involving the liver, kidney and spleen, and a squamous-celled carcinoma arose on the skin of an animal treated with 2,3,5,6-TCNB.

**INHIBITION EXPERIMENTS.** When the test compounds and BP were applied alternately, 50% of the mice bore papillomas by 21 weeks (BP alone or with 2,3,4,6-TCNB), 22 weeks (BP with PCNB), or 23 weeks (BP with 2,3,4,5- and 2,3,5,6-TCNB). When the numbers of papillomas/surviving mouse were plotted against the duration of treatment, it was confirmed that no compound had retarded BP carcinogenesis by more than 2 weeks.

In the 2nd inhibition experiment, when somewhat younger mice were used, all groups developed skin tumors earlier than in the 1st experiment. Fifty % had papillomas by 19 weeks (BP with 2,3,4,5- and 2,3,5,6-TCNB) or 20 weeks (BP alone and with PCNB and 2,3,4,6-TCNB). A similar graph again showed no significant effect of any test compound, the largest effect being an acceleration of tumor development by 1.5 weeks with 2,3,4,6-TCNB.

From these experiments it was concluded that, in the molar proportions tested (approximately 1.5 moles/mole of BP), skin

**CHART 2.** Average number of skin tumors/surviving mouse during and after treatment with croton oil. Previous treatment with acetone (——), chloroacetone (×—×), NEM (○—○), or β-BPA (●—●).
carcinogenesis by BP was not modified by the chloronitrobenzenes.

Discussion

Three of the 4 chloronitrobenzenes examined in the foregoing experiments have nitro groups that are labile in vitro and in vivo; this analogy to NQO was the main reason for carrying out the tests for carcinogenicity and inhibitory activity. The metabolism in the rabbit of all 19 members of the chloronitrobenzene series has been studied by Thorpe and his associates, with results summarized by Bray, James, and Thorpe (4). It was found that PCNB, 2,3,4,6-, and 2,3,5,6-TCNB each yield mercapturic acid by replacement of $\text{NO}_2$ to the extent of 36-37% of the absorbed dose. However, 2,3,4,5-TCNB forms no mercapturic acid, except to a small extent by replacement of chlorine. Similarly, in vitro the nitro groups of the 1st 3 compounds, but not that of 2,3,4,5-TCNB, are attacked by alkali (3).

The experiments described here have shown that all 4 compounds act as tumor initiators for mouse skin when croton oil is used as the promoting agent. Moreover, 2,3,4,5-TCNB appeared somewhat more effective than the mercapturate-forming compounds. This result discounts the importance of SH-reactivity in the activity of these compounds and suggests that, if it has a role at all, it is a protective one. The inactivity of the SH-reagent NEM in these tests and in the rat injection tests of Dickens and Jones (11) supports this view.

Probably of more importance in the observed initiation of skin tumors is the metabolic reduction of the nitro group undergone by all members of this series, at least in the rabbit (4). Hydroxylamines are formed during chemical reduction of aromatic nitro compounds (13),

$$R-\text{NO}_2 \rightarrow R-\text{NO} \rightarrow R-\text{NOH} \rightarrow R-\text{NH}_2,$$

and from at least some nitro compounds on reduction in vivo (23). They are also formed from administered amines, such as the potent carcinogen 2-acetamidofluorene (10) by $\text{N}$-hydroxylation, $R-\text{NH}_2 \rightarrow R-\text{NOH}$. The importance of this process in aromatic amine carcinogenesis has recently been critically assessed by Clayson (6).

While direct evidence is lacking, it seems possible that hydroxylamines are also formed in the skin following applications of the chloronitrobenzenes and may be responsible for the observed tumor initiatory activity. If so, some such activity might be expected in various other nitro compounds that are similarly metabolized. This mechanism may, in fact, be operative in carcinogenesis by NQO, since Shirasu (21) has shown 4-hydroxyaminoquinoline $\text{N}$-oxide to be carcinogenic on s.c. injection into rats and mice.

The present experiments have given no evidence that the chloronitrobenzenes induce tumors in the absence of a promoting agent, but the possibility that they might do so when applied in larger amounts or s.c. is being investigated.

Chloroacetone was included in our tests, as Crabtree (7) had reported that it was both an inhibitor and an accelerator of BP carcinogenesis of mouse skin, depending on the dose employed. This finding could not, however, be repeated by Argus and Arceo (1), who used acetone as the solvent in place of Crabtree's ether-paraffin mixture. Inclusion of $\beta$-BPA was suggested by its reaction with cysteine in neutral solution (17) to yield the same product, $\beta$-carboxyethylcysteine, as does the carcinogen $\beta$-propiolactone (11), and inclusion of NEM by its well-known use as an SH-blocking agent. Neither chloroacetone (7) nor $\beta$-BPA (18) had shown evidence of carcinogenicity in relatively short-term tests, but both were initiators of skin tumor development in the present experiments. It seems likely that the activity of both substances is associated with their ability to act as alkylating agents by fission of the carbon-halogen bond.

Early work on the inhibition of skin carcinogenesis, discussed in the previous papers (19, 20), showed an apparent link with mercapturate acid formation by the inhibitor in the skin. The possibility was therefore envisaged that 2,3,4,5-TCNB would not inhibit BP carcinogenesis even if the other chloronitrobenzenes tested in the present experiments were active. No inhibitory effect was, in fact, found with any of the 4 compounds examined. However, in contrast to the strong inhibitory action of NQO (19), inhibition with mercapturate-forming agents such as bromobenzene (8) and some aromatic hydrocarbons (9) needed considerably larger proportions of inhibitor to carcinogen than could be employed here, and the possibility that some chloronitrobenzenes might be inhibitory if applied in greater excess cannot be entirely excluded.

Acknowledgments

I wish to thank Dr. S. P. James for supplying samples of each of the chloronitrobenzenes examined here, and Messrs. Plant Protection Ltd. for gifts of the compounds mentioned in the experimental section. I am very grateful to Dr. A. T. Spence for reporting on a number of histologic specimens, to Dr. J. A. H. Waterhouse for carrying out the statistical evaluation of results, and to Dr. D. L. Woodhouse for valuable comments.

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Cancer Res 1966;26:12-17.

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