4-Nitroquinoline N-Oxide: An Inhibitor of Skin Tumor Initiation by 3,4-Benzpyrene

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SUMMARY
Single doses of 4-nitroquinoline N-oxide were applied to mouse skin 1, 3, or 7 days before or 1 day after application of a single dose of 3,4-benzpyrene. During subsequent croton oil treatment papillomas developed more slowly in mice treated with both carcinogens than in those receiving only the benzpyrene. The inhibition of tumor initiation was most marked with the 3-day interval and least when the benzpyrene was applied first. The dose of nitroquinoline oxide giving this inhibition was insufficient to cause severe skin damage.

Following Berenblum's discovery that applying mustard gas (2,2'-dichlorodiethyl sulfide) to mouse skin inhibited the carcinogenic action of coal tar (1,2), inhibitory activity has been found in a range of other substances. These include various aliphatic chloro-compounds (4), acid chlorides (5), acid anhydrides (7), phenols (3), and mercapturate-forming substances such as bromobenzene (6), naphthalene, anthracene, and phenanthrene (8). A number of further studies, summarized in the review by Kotin and Falk (11), have demonstrated reduction in the carcinogenic action of polycyclic aromatic carcinogens on skin by simultaneous treatment with structurally related non-carcinogenic or weakly carcinogenic compounds.

More recently, Searle and Woodhouse (17,18) found that the incidence of tumors induced in mouse skin by 3,4-benzpyrene (BP) and 1,2,5,6-dibenzanthracene (DBA) can be greatly reduced by concomitant treatment with 4-nitroquinoline N-oxide (NQO). NQO is a compound possessing a great affinity for thiol (—SH) groups, and reacts with SH-containing compounds with elimination of its labile nitro group. It had earlier been shown by Japanese workers to possess anti-fungal (13) and mutagenic (15) activity, and to be carcinogenic for the skin of mice (12) and rats (19). It has also induced tumors in the skin of the golden hamster and guinea pig (C. E. Searle, unpublished results).

Whereas in the earlier inhibition experiments the inhibitors were applied throughout the course of carcinogenesis, sometimes in massive excess as in Crabtree's experiments with bromobenzene (6), the marked inhibitory effects of NQO on skin carcinogenesis were obtained with amounts of NQO approximately equimolar with the BP or DBA. It therefore seemed possible that a specific inhibitory effect on the initiating stage of carcinogenesis might also be demonstrable with this agent. An experiment with this purpose has now been carried out and the results are described in this communication.

MATERIALS AND METHODS
Single doses of NQO were applied to mouse skin at different time intervals before and after single doses of BP. Croton oil was then applied for 20 weeks, and the rates at which papillomas developed in the various groups of mice were determined during this period and for 20 weeks afterwards. Control groups were treated with BP and croton oil, NQO and croton oil, and with croton oil only.

Compounds.—4-Nitroquinoline N-oxide was synthesized in this laboratory and purified as described by Searle and Woodhouse (18). 3,4-Benzpyrene was obtained from L. Light & Co., Colnbrook, Bucks, England. The croton oil, from Stafford Allen & Co., has been used for a number of earlier experiments in this laboratory and shows the expected tumor-promoting activity. The agents were applied in redistilled analytical grade acetone at the following strengths: NQO, 0.3%; BP, 0.2%; croton oil, 0.5%, using calibrated glass pipets.

Animals.—The experiments were made with outbred
stock white mice, used for many carcinogenicity tests in these laboratories on materials such as mineral oils (21), polycyclic hydrocarbons (18), NQO (18), etc. They were housed in galvanized boxes each containing 5 mice and were fed Thompson No. 1 cube diet and water ad libitum. Before any applications were made the mice were ear-punched for identification, and hair was removed from the whole back with electric clippers. Clipping was also carried out during the experiment at intervals of 3–4 weeks.

The influence of a single dose of a carcinogen is known to be affected by the hair cycle of the animal. A record was therefore kept of the rate of hair growth immediately following the NQO and BP applications in case any such effects should be relevant to this experiment.

Experiment.—When they were 6–7 weeks of age the mice were allocated at random to one of 7 groups, each containing 10 males and 10 females. Groups A, B, C, and D each received single applications of 0.2 ml of NQO solution and 0.1 ml of BP, followed by twice-weekly applications of 0.2 ml of croton oil for 20 weeks. The smaller volume of BP was used to ensure complete coverage by the NQO solution.

Group E received BP and croton oil but no NQO, and Group F received NQO and croton oil but no BP. Group G mice were treated with croton oil only. The time schedule of these applications is given in Table 1, Columns 2 and 3.

Skin tumors.—The papillomas present on each mouse were counted weekly during croton oil treatment and fortnightly thereafter. The results obtained at 10-week intervals in the 7 groups, excluding papillomas under 1 mm in diameter or not persisting more than 2 weeks, are given in Table 1.

The numbers of mice bearing skin tumors (Column 4) and the total numbers of papillomas (Column 5) are not cumulative and show the occurrence of regressions after ceasing croton oil treatment, particularly between 30 and 40 weeks. Papillomas on mice which had died earlier are included here but are, of course, excluded in calculating the average number of papillomas per surviving mouse. The figures thus obtained are plotted against duration of treatment in Chart 1.

The experiment was terminated 20 weeks after ceasing croton oil treatment, when there were 93 mice surviving from the original 140. The sizes of the papillomas then present were measured, and a number of those on the mice treated with NQO and croton oil only were examined histologically.

Effects of a single NQO application on mouse skin.—NQO, 0.2 ml of 0.3% in acetone, as used in the above experiment, was applied to the clipped backs of 11 young male stock white mice. These were killed singly at intervals, at first daily, then less frequently. Skin from the back of each mouse and from an untreated control mouse was fixed (4% formaldehyde-saline) and sections were stained with Ehrlich's hematoxylin eosin and Weigert's iron hematoxylin–van Gieson's stain (5).

RESULTS

Croton oil control group.—As is usually the case with experiments of this type, some papillomas developed on mice which had been treated with croton oil solution only (Group G). Some regressions occurred later in the experiment, the largest number of papillomas obtained being 15, on 7 out of 16 surviving mice, between 8 and 10 weeks after ceasing croton oil treatment.

Since the table and chart show only actual numbers of papillomas, it is worth emphasising the relatively small size of the papillomas in this group compared with many in groups A–F. At the end of the experiment no mouse in this group had a papilloma of more than 3-mm diameter.

NQO and croton oil treatment.—The control group F was in effect a test of the tumor-initiatory activity of NQO at the same dosage as given in the groups to test its inhibitory activity. Many more papillomas were obtained than with croton oil alone, a maximum of 43 being reached 6 weeks after the end of croton oil treatment. Some regressions occurred later.

When the experiment was terminated the largest papillomas in this group were 9 mm in diameter. Five of these were examined histologically and found to be simple papillomas showing no evidence of infiltration. There was a thick keratin layer and some inflammatory infiltration of the dermis. A large skin carcinoma was, however, present on 1 mouse killed at 26 weeks.

BP and croton oil treatment.—As expected, a high incidence of papillomas occurred in Group E, given a single dose of BP. These began to appear 7 weeks after starting croton oil treatment and a total of 145 was reached at the 32nd week. The surviving mice then had an average of over 8 papillomas/mouse; the sharp drop shown by this group (Chart 1) between 28 and 30 weeks is due to the death of 1 mouse bearing 19 papillomas. At the end of the experiment 23 of the remaining 88 tumors were over 5-mm diameter and 8 over 10-mm.

Of the 3 animals in this group which did not develop skin tumors, 1 was killed early in the experiment with a disease unassociated with the treatment, and another died

### Table 1

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NQO APPLIED ON DAY</th>
<th>BP APPLIED ON DAY</th>
<th>MICE WITH TUMORS AT WEEKS</th>
<th>TOTAL TUMORS AT WEEKS</th>
<th>DISEASE UNASSOCIATED WITH TREATMENT</th>
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<td>4</td>
<td>15</td>
<td>42</td>
</tr>
<tr>
<td>B</td>
<td>-3</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>34</td>
</tr>
<tr>
<td>C</td>
<td>-1</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>48</td>
</tr>
<tr>
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<tr>
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<td>-1 (None)</td>
<td>2</td>
<td>11</td>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>G</td>
<td>(None)</td>
<td>2</td>
<td>6</td>
<td>7</td>
<td>12</td>
</tr>
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</table>

*From start of croton oil treatment on Day +3.

End of croton oil treatment.

Surviving mice at 20 weeks: Group A, 20; B, 18; C, 17; D, 18; E, 17; F, 19; G, 17.

Surviving mice at 40 weeks: Group A, 13; B, 14; C, 13; D, 11; E, 12; F, 14; G, 16.

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CHART 1.—Average number of papillomas per surviving mouse during and after croton oil applications. Previous treatment: Groups A, B, and C, NQO before BP; Group D, NQO after BP; Group E, BP only; Group F, NQO only; Group G, none. End of croton oil treatment indicated by vertical line.

Effects of NQO on tumor initiation by BP.—All 4 groups of mice (A—D) treated with NQO in addition to BP developed fewer skin tumors than did those receiving BP only prior to croton oil treatment. The time interval from start of treatment to 50% tumor incidence in the mice at risk increased by the NQO from 10 weeks (Group E) to 14—18 weeks (A—D), as shown in Table 1, Column 6.

The effect was least marked in Group D, treated with NQO 1 day after BP. At the end of croton oil treatment there were 31% fewer tumors in this group than with BP alone (Group E), and in later weeks the difference became more marked owing to the continued development of tumors in the latter group only.

NQO applied 1 day before BP (Group C) had a greater effect. The appearance of the first papilloma was delayed for 4 weeks, and at 20 weeks there were 55% fewer papillomas as a result of NQO application.

The most marked effect was obtained when NQO was applied 3 days before BP (Group B). The first papilloma arose 6 weeks later than in Group E, and the reduction in number of papillomas at 20 weeks was 78%. It is surprising that the incidence of papillomas obtained in this group was smaller than in Group F with NQO only, as if BP were actually protecting against tumor initiation by NQO.

When the time interval between NQO and BP was increased to 7 days (Group A), the inhibitory effect of NQO on BP tumor initiation was considerably reduced compared with Group B. Nevertheless, it was still considerably greater than that of NQO applied after BP (Group D) except towards the end of the experiment.

Statistical assessment of results.—For convenience of estimation, the tumors present in groups A—E were assessed, by means of analysis of variance, at 10-weekly intervals after the initial treatment with croton oil.

When total tumors were assessed, the differences in incidence between the control group E and each of the NQO-treated groups A—D were statistically significant at all time intervals. This was also true when tumors per survivor were assessed, except that the difference between D and E was then not significant at 20 weeks. This is due to a high variance in response shown by these groups, particularly at 20 and 30 weeks.

As the effects of NQO appeared greater in group B than in groups A, C, and D, the differences between A and B...
were also assessed. These were found to be significant at 30 and 40 weeks taking all tumors into account, but at 30 weeks only taking tumors per survivor.

Hair-growth phase of treated mice.—The numbers of mice in each group showing appreciable hair growth in the week preceding croton oil treatment were: A, 4; B, 1; C, 1; D, 1; E, 1; F, 2; G, 1. The single mice in Groups B and E, and 1 of the 4 in Group A, were the only animals in their respective boxes not to develop papillomas. This suggests that these animals, because of hair growth at the relevant time, received a lower effective dose of BP than the other mice, but their numbers are not such as to affect materially the results of the experiment.

Skin changes following a single application of NQO.—The histology of mouse skin, taken at intervals of 1–51 days after the NQO application, was studied, but the changes observed were not pronounced. The most noticeable effects were enlargement and slight proliferation of the epithelial cells most marked at 2 and 3 days after NQO application, with pronounced mitotic activity at 3 days. In the dermis, inflammatory changes were maximal at 1 and 2 days. The keratin layer was appreciably thicker than the control from about the 4th until the 30th day. The hair follicles and sebaceous glands appeared unaffected at all time intervals.

DISCUSSION

The process of carcinogenesis is often regarded as consisting of 2 main stages, initiation and promotion. Initiation is a rapid process with apparently permanent results, while promotion is a prolonged process (see review by Salaman and Roe, 16).

Where inhibiting substances have been applied throughout the course of carcinogenesis by an agent such as BP it is not possible to say whether the inhibition occurs at any specific stage of carcinogenesis. Tannenbaum has, however, demonstrated (20) that caloric restriction can modify the process of promotion but not that of initiation, and successful inhibition of initiation does not appear to have been reported to date (16).

The experiment described in this paper has now shown that the effect of a single initiating dose of BP on mouse skin can be very markedly reduced by a single dose of another carcinogen, NQO, applied 1, 3, or 7 days before or 1 day after the BP. At the most effective time interval studied, with the NQO given 3 days before the BP (Group B), surviving mice after 20 weeks of croton oil treatment had an average of only 1.2 papillomas each, compared with an average of 6.2 in mice treated identically except for the NQO application (Group E). When the NQO preceded the BP by 1 or 7 days (Groups C, A) the effects of the NQO, though reduced compared with Group B, still represented reductions of tumor incidence of more than 50% at the end of croton oil treatment.

A marked reduction in the incidence of papillomas was also found in the 1 group (D) treated with NQO after BP. If initiation is indeed a completely irreversible process this would indicate that initiation, though rapid compared with promotion, is not complete 24 hr after application of the BP. Similar experiments using different time intervals and carcinogens might give useful information on the duration of the initiatory process.

If experiments of the type reported here are to be taken at their face value as indicating direct interference with tumor initiation, other possible less direct explanations of the effects observed must be excluded. Destruction by NQO of cells which would otherwise undergo tumor initiation by BP would, for instance, be expected to lead to a reduced incidence of tumors.

The results from a small-scale experiment to test this point did not, in fact, show any very severe effects attributable to treatment with NQO under the conditions used in the inhibition experiment (see "Results"). This is in contrast to the results reported by Hayashi (10), who examined the skin of DD strain mice at intervals from 1 to 48 hr following application of NQO in acetone or benzene. Within 24 hr he observed intra-epidermal vesicles or cleavages, associated with degenerative changes of the epidermal cells and severe exudative reaction in the dermis.

Two factors may be responsible for the greater effects of NQO found by Hayashi. Firstly, though the concentration of NQO (0.25%) was slightly less than we have used, the volume applied and the area of interscapular skin treated were not specified, and the dose of NQO per unit area of skin may well have been greater. Secondly, the differences may be attributable to the use of different mouse strains. In experiments to be reported shortly, we have found very marked differences in response to NQO applications between a number of mouse strains bred in these laboratories, in particular C57B1, IF, NZY, and Strong A mice.

Another possible indirect explanation for the results in the inhibition experiment is that NQO treatment reduces the effective dose of BP to the skin. Increased secretion from the sebaceous glands washing out the carcinogen would provide one way in which this could occur, but would have to be considerable to account for the degree of inhibition observed, particularly at the 3-day interval.

The thickness of the keratin layer does not seem to be a significant factor, since this was greatest when the inhibitory effect was already becoming weaker.

The most important change in the treated skins may have been the pronounced mitotic activity observed in the epithelial cells at 3 days, the time when the greatest inhibition of tumor initiation was obtained. For any further elucidation of these points it is evident that a more detailed study of the effects of NQO application is required, and this is now in progress. In any event, the markedly greater effect of the 3-day interval compared with the 1-day interval indicates that the inhibitory effect of NQO is not a direct result of the blocking of SH-groups, since even the non-enzymic interaction between SH-groups and NQO is a very rapid one.

NQO and some closely related compounds are unusual in having such labile nitro groups and in the readiness with which these react, apparently specifically, with thiol-containing compounds (9, 14, 17). The nitro groups of some chlorinated nitrobenzenes are known, however, to be labile to a lesser extent, being replaceable on hydrolysis in vitro and during mercapturic acid formation in vivo.

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Comparative tests for carcinogenic and inhibitory activity in some of these compounds have been carried out and will be reported shortly.

ACKNOWLEDGMENTS

I am very grateful to Dr. A. T. Spencer for reporting on the histology of the NQO-treated mouse skins, to Dr. J. A. H. Waterhouse for the statistical calculations, and to Dr. M. H. Salaman for valuable comments on the results of the inhibition experiment with NQO.

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