4-Nitroquinoline N-Oxide: An Inhibitor of Benzpyrene Carcinogenesis of Mouse Skin*

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SUMMARY

The rate of appearance of papillomas on the skin of mice treated with 3,4-benzpyrene was markedly retarded by 4-nitroquinoline N-oxide, applied either in the same solution as the benzpyrene or 3 days before each benzpyrene application. Carcinogenesis by 1,2,5,6-dibenzanthracene was also inhibited. No inhibition was observed when the nitroquinoline oxide and benzpyrene were applied to separate sites on the mice. The significance of the results is discussed with regard to the toxicity of 4-nitroquinoline N-oxide and the known inhibition of skin carcinogenesis by other agents which interact with sulfhydryl groups.

The chemistry and biology of compounds derived from the N-oxides of pyridine and quinoline have been extensively investigated by Japanese workers (21). A compound of particular interest biologically is 4-nitroquinoline N-oxide (NQO), which was shown to have antifungal and mutagenic activity by Okabayashi (22, 24) and later to be carcinogenic for mouse skin by Nakahara, Fukuoka, and Sugimura (20) and for rat skin by Takayama (28).

We have confirmed the carcinogenicity of NQO (26, 27) but, like Lacassagne, Buu-Hoï, and Zajdela (17), lost many mice during the experiments owing to the general toxicity of the compound. The antitumor effects of NQO, reported by Sakai et al. (25) and by Moore et al. (18), are thought by the latter authors to be due to this general toxic effect.

The nitro group of NQO is very labile, being substituted by nucleophilic centers with elimination of nitrite. Okabayashi (23) and Endo (15) showed spectrophotometrically that ready interaction occurs with thiol compounds, including such physiological constituents as cysteine and glutathione, in neutral aqueous solution at room temperature. Under similar conditions no reaction could be demonstrated between NQO and various amino acids, nucleotides, or nucleic acids. We tested a range of compounds (26, 27) using the diazo and starch-iodine reagents to detect liberated nitrite, and also found that compounds containing a free —SH group readily reacted with NQO in buffer at pH 7. There was no detectable reaction with various sulfur-free compounds, disulfides, thioethers, or even with thiourea derivatives possessing a potential —SH group.

When we incubated NQO with slices or homogenates of various rat and mouse tissues, the amount of NQO taken up from the medium corresponded reasonably well with that expected for reaction with tissue —SH groups, on the basis of the —SH concentrations determined for these tissues by Calcutt and co-workers (6-8) using p-chloromercuribenzoic acid as the —SH reagent, though direct comparisons between the uptake of NQO and of a recognized —SH reagent in the same sample would of course be desirable.

The evidence that chemical carcinogens induce a rise in —SH levels in the target tissues and that compounds which block —SH groups or form mercapturates reduce tumor yields has been summarized by Calcutt (5). Since NQO is an avid reagent for —SH groups it was thus of interest to test its effects upon carcinogenesis by polycyclic hydrocarbon carcinogens. That alternate applications of NQO markedly reduced the yield of papillomas in mice after 20 weeks of treatment with 3,4-benzpyrene (BP) was reported at the 8th International Cancer Congress in Moscow in July, 1962 (26, 27), and the present paper records the results of this experiment to its conclusion after 30 weeks of applications. Other experiments reported here are concerned with the effects of simultaneous applications of NQO upon skin carcinogenesis by BP and 1,2,5,6-dibenzanthracene (DBA), and of applying NQO and BP to different sites of the same mouse.

MATERIALS AND METHODS

Compounds.—4-Nitroquinoline N-oxide was prepared by Ochiai’s method (21) as modified by Moore et al. (18). (We are indebted to Dr. G. Fare for this material.) It was found that a “cleaner” yellow product of a slightly higher melting point (155°C.) could be obtained by crystallization from benzene-light petroleum, followed by ethanol. This purification appears to be due to removal

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of some benzene-insoluble tarry material, probably derived from decomposition of the nitrated quinoline oxide during washing with sodium carbonate solution. Since NQO is very sensitive to alkali, it is probably better to omit the wash with dilute sodium carbonate completely.

3,4-Benzyopyrene and 1,2,5,6-dibenzanthracene were obtained from L. Light & Co., Colnbrook, Bucks. The compounds were applied in Analar acetone at a concentration of 0.2 per cent (BP) or 0.3 per cent (NQO, DBA).

Animals.—Hair was removed from an area in the mid-back of young, adult stock white mice with electric clippers at the start of each experiment only. The test solution (0.2 ml.) was applied to this area from a glass pipet at the times specified in the "Results" section. The times of appearance of macroscopic papillomas and the number of papillomas per mouse were recorded, usually weekly.

In the "two-site" experiment two smaller areas of back, each about 1 cm. in diameter, were used, one at the back of the neck and the other just in front of the tail. The volume applied to each patch had to be reduced to 0.1 ml. to ensure against one solution's overlapping the other. The solutions were applied weekly, one immediately after the other.

The numbers of mice and papillomas given in the tables are cumulative—i.e., they include mice which had died or had been killed by the times stated. No distinction is made between male and female mice, since no significant differences in tumor yield were observed.

RESULTS

Alternate applications of NQO and BP.—One group of 25 mice was given applications of NQO on Mondays and

### TABLE 1

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<tr>
<th>Weeks</th>
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<th>NQO + BP, alternately</th>
<th>NQO + BP, together</th>
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### TABLE 2

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of BP on Thursdays. The rate of appearance of papillomas was compared with the rates obtained in similar groups treated at the same time with the same amount of NQO and BP separately. The results are summarized in Table 1.

It was found that NQO applied alternately with the BP caused a delay of some 4 weeks before the appearance of the first papilloma. The subsequent rate of development of papillomas was markedly reduced, whether reckoned on the number of mice bearing papillomas or on the total number of papillomas obtained. After 26 weeks the individual tumors on the BP control mice were merging and could not be counted. Twenty mice (80 per cent of those “at risk”) had tumors at this time; in the mice treated alternately with NQO the number was six (27 per cent).

Simultaneous application of NQO and BP.—In another experiment NQO and BP were applied weekly to mouse skin in the same quantities as before, but in a single solution instead of separately. Examination of the U.V. spectra of the separated and diluted constituents indicated that there was no significant loss of NQO or BP on storage at 4° C.

The results of this experiment are also given in Table 1. Separate control groups were not run with this test, but it is nevertheless clear that the incidence of papillomas was again greatly reduced by the NQO. In this experiment, as with NQO alone, a few mice developed tumors after a relatively short period of treatment.

If the numbers of tumor-bearing mice, as percentages of those “at risk” (i.e., alive at the time the first papilloma was observed) are plotted against duration of treatment, the curve obtained for the mice treated with the carcinogens in the same solution is not very different from that for the NQO control mice. That for the mice treated with BP and NQO separately suggests that, once tumors

**TABLE 3**

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have started to appear, BP carcinogenesis is somewhat less efficiently inhibited. However, such curves do not allow for the greater number of mice which died tumor-free at later stages of the experiments when both substances were applied.

Simultaneous applications of NQO and DBA.—In order to see whether carcinogenesis by a different polycyclic hydrocarbon would be similarly inhibited by NQO, 30 mice were given weekly applications of an acetone solution containing 0.3 per cent each of NQO and DBA (0.2 ml.), and the results were compared with those from control mice treated at the same time with a solution containing the same concentration of DBA only (Table 2).

The rate at which mice developed papillomas with this hydrocarbon, slower than that with BP, was also retarded by simultaneous treatment with NQO. A few early papillomas were again found on mice treated with both carcinogens. However, as with BP and NQO, the combined treatment resulted in a higher mortality than that found in the control mice.

"Two-site" experiment.—The reduction in the amount of BP applied to the control animals in this experiment resulted in a much slower tumor production on both front and rear sites (Tables 3, 4). The yield of BP tumors on the rear site was not affected by the application of NQO to the front site, where, however, some additional papillomas appeared, one after only 5 weeks of treatment. Application of NQO to the rear site also caused no inhibition of BP tumor development at the front site; in fact, in this case there appeared to be a slight enhancing effect. No papillomas appeared at the rear NQO site.

DISCUSSION

Many substances have been studied for their effects on experimental skin carcinogenesis. As long ago as 1929 Berenblum (1, 2) found that mustard gas (2, 2′-dichlorodiethyl sulfide), which he expected to enhance the carcinogenicity of coal tar, actually had a marked inhibitory action. The effect could not be ascribed to any interaction of mustard gas with active constituents of the tar or to a "super-optimal" degree of irritation of the skin, and it was necessary to assume a specific chemical effect of the inhibitor on the tissues. He later showed (3) that carcinogenesis by a pure chemical carcinogen, DBA, could be similarly inhibited and that, although a number of other irritants had inhibitory activity like that of mustard gas, there were many irritants without such activity.

Looking for a biochemical basis for these results, Berenblum, Kendal, and Orr (4) found that most but not all inhibitory compounds suppressed glycolysis more than respiration in Jensen rat sarcoma tissue, whereas non-inhibitory compounds did not do so. Crabtree, however (9), showed that the inhibitory activity of a range of aliphatic chloro-compounds could also be correlated with the reactivity of the Cl atom, as measured by its rate of reaction with cysteine. The most active of these compounds was chloroacetone; but, whereas it showed marked activity applied in a 0.3 per cent solution, the effect was lost when the strength was increased to 3 per cent, and in animals pretreated with BP chloroacetone actually accelerated the rate of tumor production (10).

When a number of acid chlorides were also shown to be inhibitory (11), Crabtree considered that the inhibition, both of carcinogenesis and of glycolysis, could be attributed to nonspecific cell damage by HCl liberated on hydrolysis, but the involvement of tissue sulfur chemistry became evident when it was found that skin carcinogenesis could be inhibited by mercapturate-forming compounds such as bromobenzene (12), naphthalene, anthracone, and phenanthrene (14). All these inhibitors caused marked falls in skin glutathione levels and the expected changes in the urinary sulfur content, indicating that the first stage at least of mercapturate formation occurred in the skin. Inhibitory activity of some dibasic acid anhydrides (13) was also explained in terms of reaction between the anhydrides and SH-containing skin constituents.

No evidence of mercapturate formation in skin was obtained when BP and DBA were applied, nor have mercapturates been demonstrated following administration of polycyclic hydrocarbon carcinogens by other routes. Nevertheless, various observations connect the effects of carcinogenic as well as noncarcinogenic hydrocarbons on growth with the metabolism of sulfur-containing amino acids. For example, White and White (29) showed that the inhibition of rat growth by 20-methylcholanthrene, BP, and pyrene could be promptly reversed by administration of cystine, cystine disulfoxide, or methionine.

There are a number of reports in the literature concerning the anticarcinogenic effects of various weakly carcinogenic or inactive compounds structurally related to the strongly carcinogenic hydrocarbons (see, for example, the relevant section in the review by Kotin and Falk [16]). The difficulties of elucidating the interaction of such compounds with tissue constituents appear similar to those occurring with the carcinogens themselves, and this paper is concerned rather with inhibitory substances, such as NQO, which possess a more clearly defined chemical reactivity.

With its ready and selective reactivity with SH-containing compounds (15, 23, 26, 27), NQO would seem a particularly interesting compound for investigating biochemical changes involved in carcinogenesis. Not only is it carcinogenic for some species of rodent (20, 26–28), but the experiments reported here also show that it markedly inhibits tumor induction in mouse skin by BP. Moreover, the effect of NQO applied 3 days before the BP was similar to its effects when both compounds were applied in the same solution, contrasting strongly with the very short-term effects of bromobenzene, at least on skin glutathione content (12). The inhibitory effect of NQO was also marked, though less striking with DBA, which in control mice induced papillomas more slowly than did BP despite its higher concentration.

It is difficult to compare directly the inhibitory effects of NQO observed in these experiments with those already reported for, e.g., mustard gas (1–3) or chloroacetone (10). Mustard gas was tested mainly as an inhibitor of carcinogenesis by tar, whereas in Crabtree's experiments (10) the solutions of BP were applied twice weekly and those of chloroacetone 3 or 6 times weekly. In our experiments single weekly applications of each compound were given.
Also, although the rate of tumor induction in mice treated simultaneously with a hydrocarbon carcinogen and NQO was reduced, we also found, as in our NQO carcinogenicity tests, that a few mice developed papillomas early, suggesting that these were induced by the NQO.

When we tested NQO for skin carcinogenicity by applying 0.3 ml of a 0.3 per cent solution twice weekly to either stock white or C57BL × IF hybrid mice, many animals became emaciated and died before producing tumors (unpublished experiments). We have also found skin applications of NQO to be followed rapidly by toxic symptoms in rats and more slowly in cayus. The smaller amounts of NQO applied in the inhibition tests reported here were not accompanied by noticeable toxic effects, and the animals appeared to be in no worse condition when treated with NQO together with BP or DBA than with the carcinogenic hydrocarbons alone. Nevertheless, it was found that more animals died tumor-free during the course of these experiments in groups treated with both NQO and hydrocarbon, which raises the question whether at least part of the inhibition should be ascribed to the toxic action of NQO absorbed into the system, rather than to direct action at the site of application.

The two-site experiment was carried out in an attempt to get some more information on this point. The experiment could not unfortunately be comparable to the other inhibition tests, since it was necessary to reduce the volumes of BP and NQO solutions applied to the smaller areas of skin used. The rate of tumor induction in the BP control mice was, as a result, markedly reduced—to a lower level, in fact, than when we have made comparable applications to larger areas of regularly clipped skin. However, there was no noticeable inhibition of BP tumor induction by the NQO applied at a separate site, despite the occurrence again of a few more tumor-free deaths in mice treated with both compounds.

There was, in fact, some indication that NQO applied at the rear site enhanced the carcinogenic effect of BP at the front site. This is in line with experiments showing that NQO can exert an additive carcinogenic effect on 20-methylcholanthrene carcinogenesis of mouse skin (19) and on 4-dimethylaminobenzene carcinogenesis of rat liver (28) when only minimal amounts of each substance are administered. A more facile removal and ingestion of NQO from the rear site as a result of licking might thus explain both the slight enhancement of BP carcinogenesis at the front site only, and the appearance of several tumors at the front NQO site whereas none appeared at the rear.

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