

November 30, 2004

Barbara Shane  
NTP Executive Secretary  
National Institute of Environmental Health Sciences  
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111 T.W. Alexander Drive  
Research Triangle Park, NC 27709

Dear Dr. Shane:

Our company has recently been made aware of several letters written by Mrs. Linda Beckett that have been posted on the NTP website, as well as comments made by Mrs. Beckett to a meeting with the NTP Board of Scientific Counselors. These letters and comments can, at best, be viewed as unfavorable to Airepel, its parent Arkion Life Sciences and our EPA-approved product lines FlightControl® Plus and Avipel®. There are several inaccurate statements made by Mrs. Beckett which could confuse and mislead the Board. We would like to address some of these inaccuracies and reassure the NTP and its Board of our responsible care with this chemical and the safe application of all our products. Mrs. Beckett offered her opinions; however, good decisions require sound science based on factual information.

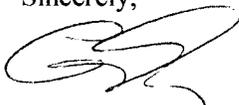
States are not “further tightening EPA restrictions” as stated by Mrs. Beckett. New York adopted the same language as approved by the USEPA. They restricted the use on school grounds to avoid contact by children because of the NTP study. New York is now reviewing their position in light of the peer-reviewed, published studies (attached). Every other state recognizes the federal language and allows applications to a full range of turf sites. There is no label in California because there is no goose pressure on turf that justifies the expense of the application.

Avipel sales are restricted to state licensed pest control operators only and available from one licensed distributor. The label is regulated by the USEPA. We have limited the availability to 28 states. Regarding application of our products, our company’s policy is to follow the law as stated by 40 CFR. Ignoring product warnings or not following the law by employees or distributors would be grounds for a 6.a.2 filing and termination.

Regarding her statement that AQ vapor lingered “for as much as 11 days,” the vapor pressure of AQ is  $1.6 \times 10^{-7}$  mm HG at 25 C. There is no vapor or odor let alone a residual of 11 days.

Airepel and NTP share similar goals with this issue: use good science for good decisions. NTP made the correct scientific decision with regard to the science surrounding AQ in February 2004 and we support that decision.

We request that this letter (with attachments) be forwarded to each member of the Board prior to the meeting in December.

Sincerely,  
  
Chris Widrig

Attachments



# Contamination Is a Frequent Confounding Factor in Toxicology Studies with Anthraquinone and Related Compounds

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Anthraquinone (AQ) (9,10-anthracenedione) is an important compound in commerce. Many structurally related AQ derivatives are medicinal natural plant products. Examples include 1-hydroxyanthraquinone (1-OH-AQ) and 2-hydroxyanthraquinone (2-OH-AQ), which are also metabolites of AQ. Some commercial AQ is produced by the oxidation of anthracene (AQ-OX). In the recent past, the anthracene used was distilled from coal tar and different lots of derived AQ often contained polycyclic aromatic hydrocarbon contaminants, particularly 9-nitroanthracene (9-NA). Many toxicology studies on AQ used contaminated anthracene-derived AQ-OX, including a National Toxicology Program (NTP) 2-year cancer bioassay that reported a weak to modest increase in tumors in the kidney and bladder of male and female F344/N rats and in the livers of male and female B6C3F<sub>1</sub> mice. The AQ-OX used in that bioassay was mutagenic and contained 9-NA and other contaminants. In contrast, purified AQ is not genotoxic. The purpose of this paper is to provide additional information to help interpret the NTP cancer bioassay. This paper describes a quantitative analytical study of the NTP anthracene-derived AQ-OX test material, and presents the results of mutagenicity studies with the 1-OH-AQ and 2-OH-AQ metabolites and the primary contaminant 9-NA. Purified 1-OH-AQ and 2-OH-AQ exhibited only weak mutagenic activity in selected strains of tester bacteria and required S9. Literature reports of potent mutagenic activity for 1-OH-AQ and 2-OH-AQ in bacteria minus S9 are, once again, very likely the result of the presence of contaminants in the test samples. Weak activity and limited production of the 1-OH-AQ and 2-OH-AQ metabolites are possible reasons that AQ fails to exhibit activity in numerous genotoxicity assays. 9-NA was mutagenic in tester strains TA98 and TA100 minus S9. This pattern of activity is consistent with that seen

with the contaminated AQ-OX used in the NTP bioassay. Analysis of all the mutagenicity and analytical data, however, indicates that the mutagenic contamination in the NTP bioassay probably resides with compounds in addition to 9-NA. 9-NA exhibited potent mutagenic activity in the L5178Y mammalian cell mutagenicity assay in the presence of S9. The positive response was primarily associated with an increase in small colony mutants suggesting a predominance of a clastogenic mechanism. Quantitative mutagenicity and carcinogenicity potency estimates indicate that it is plausible that the contaminants alone in the NTP AQ-OX bioassay could have been responsible for all of the observed carcinogenic activity. Although AQ-OX is no longer commercially used in the United States, many of the reported genotoxicity and carcinogenicity results in the literature for AQ and AQ derivative compounds must be viewed with caution.

**Keywords** Anthraquinone, Contamination Issues, 1-Hydroxyanthraquinone, 2-Hydroxyanthraquinone, Mutagenicity

Anthraquinone (AQ) (9,10-anthracenedione) and structurally related compounds are important in commerce and many are found as natural plant products. AQ is used as an intermediate in the manufacture of dyes and to enhance the efficiency of the Kraft process for the production of paper, thus reducing the number of trees harvested (Cofrancesco 1992). AQ is the active ingredient in the most effective and nonharmful bird repellent used for keeping birds from airport runways or areas where they would conflict with the human population (Ballinger and Price 1996; Cummings et al. 1997; Ballinger, Gilmore, and Price 1998; Dolbeer et al. 1998). AQ derivatives are widely found in plants. Hydroxyanthraquinones are the biologically active components of many phytotherapeutic drugs, including plant-derived laxatives such as aloe, senna, frangula, emodin, and rheum. Uses also include treatments for kidney and bladder stones, and as a mild sedative (Tikkanen, Matsushima, and Natori 1983; Westendorf et al. 1990).

Reports of the activity of AQ and related compounds in genetic toxicology and carcinogenicity assays are decidedly

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mixed, with many examples of opposite results reported from different laboratories. A critical review of the literature shows that in many cases, contamination with mutagenic compounds is the probable reason for the contrasting results. It is common that the focus of a publication will be on one or a set of AQ derivatives found naturally in plants, yet the test material for the genetic toxicology or cancer studies will be a synthetic product with the potential for containing biologically active contaminants (Tikkanen et al. 1983; Kawai et al. 1986; Westendorf et al. 1990; Mori et al. 1990, 1992; NTP 2004).

From a toxicological viewpoint, a recurring problem in studies reported earlier is that a formerly common synthetic pathway to produce AQ was from the oxidation of anthracene (AQ-OX). The anthracene used was distilled from coal tar and different lots of derived AQ contained varying amounts of polycyclic aromatic hydrocarbon contaminants, particularly the mutagenic isomers of nitroanthracene (Cofrancesco 1992; Butterworth, Mathre, and Ballinger 2001). In a 2-year study the National Toxicology Program (NTP) reported that anthracene-derived AQ-OX induced a weak to modest increase in tumors in the kidney and bladder of male and female F344/N rats and a modest increase in the livers of male and female B6C3F<sub>1</sub> mice (NTP 2004). Further analysis of that study showed that the anthracene-derived test material was contaminated with 9-nitroanthracene as well as other polycyclic aromatic hydrocarbons and was also mutagenic in bacterial assays (NTP 2004; Butterworth, Mathre, and Ballinger 2001). When the test material was purified, the pure AQ was found to be without mutagenic activity (Butterworth, Mathre, and Ballinger 2001). In contrast to most reports, however, the NTP noted that the study material was anthracene-derived AQ-OX and acknowledged the contamination issue. Thus, although the NTP bioassay is a valid study that directly applies to exposure to AQ-OX, the degree to which pure AQ may be carcinogenic is not known. The main purpose of the analytical and genotoxicity studies reported in this paper was to provide additional information to help with the interpretation of that NTP bioassay. In the course of this evaluation, it became evident that mutagenicity and carcinogenicity studies with some of the AQ derivatives were also likely affected by contamination as a result of the particular synthetic pathways used in their preparation.

In assessing the potential biological activity of preparations of AQ, it is critical to be aware of how the preparation of interest was manufactured and the potential contaminants inherent with the different synthesis processes. AQ is produced in large quantities by three different production methods in various parts of the world (Cofrancesco 1992). The oxidation of anthracene to yield AQ is practiced primarily in Europe, but is now in declining use. AQ from the oxidation process (AQ-OX) involves the oxidation of anthracene derived from coal tar. Distillates that carry over in the same fraction as anthracene often remain as contaminants in the final anthracene-derived AQ-OX product. These include a variety of polycyclic aromatic hydrocarbons with similar boiling points. For example, 9-nitroanthracene and anthracene distill within 5 degrees of each other.

The AQ-OX contamination problem has been known for some time and profiles of contaminants from this process can differ substantially. Of particular concern is the observation that the mutagenic nitroanthracenes are often seen in AQ-OX preparations, sometimes at concentrations over 2.5% (U.S. EPA 1977; ICI 1978a, 1978b). An illustration of the variability between lots is illustrated by comparing the anthracene-derived AQ-OX used in the NTP cancer bioassay to that of the anthracene-derived AQ-OX used in the companion NTP mutagenicity studies (NTP 2004). The actual material used in the 2-year cancer bioassay has a reported contaminant level in the range of 0.1% to 0.65%, depending on the analytical testing laboratory, and is a bacterial mutagen. Material used in the companion NTP mutagenicity studies had a contaminant level of 3% and was highly mutagenic. In both cases, when the anthracene-derived AQ-OX was purified and retested, no mutagenic activity was observed for the pure AQ (Butterworth, Mathre, and Ballinger 2001; NTP 2004).

Benzene and phthalic anhydride undergo the Friedel-Crafts reaction to yield *o*-benzoylbenzoic acid, which is treated with concentrated sulfuric acid to yield AQ (Cofrancesco 1992). This is the most prevalent production method employed in China and India. AQ produced by the Friedel-Crafts process (AQ-FC) is not a bacterial mutagen and is substantially free of the polycyclic aromatic contaminants and nitroanthracenes that are often found in AQ-OX (Butterworth, Mathre, and Ballinger 2001).

Production of AQ by the Diels-Adler reaction (AQ-DA) between 1,4-naphthoquinone and 1,3-butadiene is practiced primarily in Japan. Because this process involves shifts between the aqueous and organic phases, contaminants are easily removed and AQ-DA is particularly clean and free of contaminants and is not mutagenic in a variety of genetic toxicology test systems (Butterworth, Mathre, and Ballinger 2001).

All anthraquinone used commercially in the United States today is AQ-FC or AQ-DA, rather than AQ-OX. Unfortunately, chemical supply houses such as Zeneca Fine Chemicals and Aldrich Chemical Company regularly stocked anthracene-derived AQ-OX at the time that the studies were begun by the NTP.

A revealing demonstration as to the variability in purity of different preparations of AQ with corresponding opposite conclusions as to genotoxic activity is to critically examine the published literature for this compound. Negative results in the Ames *Salmonella* bacterial mutagenicity assay have been reported by nine independent laboratories (Brown and Brown 1976; Anderson and Styles 1978; Gibson, Smart, and Smith 1978; Salamone, Heddle, and Katz 1979; Sakai, Yoshida, and Mizusaki 1985; Tikkanen, Matsushima, and Natori 1983; National Cancer Institute 1987; Butterworth, Mathre, and Ballinger 2001; NTP 2004). In contrast, positive mutagenicity assays were reported from two laboratories that used anthracene-derived AQ-OX (Lieberman et al. 1982; Zeiger et al. 1988). The pattern of activity of AQ reported from the AQ-OX studies was also unusual in that mutagenic activity was seen without metabolic activation. The chemical structure of AQ does not suggest that the parent

compound would be a DNA-reactive mutagen. Comparative studies show that a mutagenic contaminant was present in the positive Ames test samples that was either directly mutagenic or could be activated by bacterial metabolism (Butterworth, Mathre, and Ballinger 2001; NTP 2004).

Similar conflicting results were observed with micronucleus assays. AQ-DA tested negative in the *in vivo* mouse micronucleus assay (Butterworth, Mathre, and Ballinger 2001). In contrast, using contaminated anthracene-derived AQ-OX, the NTP reported a weak, positive response in a mouse peripheral blood micronuclei assay test from the 14-week range-finding study that preceded the cancer bioassay (NTP 2004). Even though the doses used were up to 4 times of the maximum tolerated dose used in the bioassay, no response was seen in the female animals, and the response in the males was judged as positive only with a highly nonconservative trend test. In contrast, anthracene-derived AQ-OX administered by intraperitoneal injection was negative in a bone marrow micronucleus assay (NTP 2004). Similarly, there is one report of weak induction of micronuclei in SHE cells, but the material used was again the contaminated NTP anthracene-derived AQ-OX (Gibson et al. 1997). The experimental evidence indicates that pure AQ does not induce micronuclei.

A characteristic of a nongenotoxic compound is demonstrated lack of activity in a variety of *in vivo* and *in vitro* genetic toxicology assays with different end points. In addition to testing negative in bacterial mutagenesis assays and the *in vivo* mouse micronucleus assay, AQ tested negative in the chinese hamster ovarian (CHO) cell chromosomal aberration assay (Butterworth, Mathre, and Ballinger 2001), the L5178Y mouse lymphoma forward mutation assay (Butterworth, Mathre, and Ballinger 2001), an assay assessing mutagenic activity in a line of human B-lymphoblastoid cells that constitutively express cytochrome P4501A1 (Durant et al. 1996), and in the Syrian hamster embryo (SHE) cell transformation assay (Kerckaert et al. 1996). This extensive testing battery clearly demonstrates that pure AQ is not genotoxic.

1-Hydroxyanthraquinone (1-OH-AQ) and 2-hydroxyanthraquinone (2-OH-AQ) are metabolites of AQ (NTP 2004). 1-OH-AQ is present in some plant preparations as the glycoside. Blomeke et al. (1992) reported that 1-OH-AQ was positive in the Ames mutagenicity bacterial tester strain TA1537 without S9. In contrast, the NTP found 1-OH-AQ to be without mutagenic activity in the bacterial mutagenicity assays they conducted (NTP 2004). The NTP, however, reported that 2-OH-AQ was a potent bacterial mutagen in strain TA98 without S9 (NTP 2004). Of concern with these studies is that neither 1-OH-AQ nor 2-OH-AQ appear to have the structure of a direct acting DNA-reactive mutagen and, thus, reports of activity without S9 are suspect. Mori et al. (1990) found that 1-OH-AQ induced tumors in the large bowel, liver, and stomach of treated rats. Kawai et al. (1986) reported that 1-OH-AQ induced DNA repair in primary rat hepatocytes. Interestingly, mechanistic studies indicate that the 1-OH-AQ-induced large bowel tumors are likely pro-

duced to a large extent via a nongenotoxic-cytotoxic mode of action resulting from biological activity associated with high-dose, chemically induced severe inflammation, crypt abscesses and erosion, and ulcerative colitis in the colonic mucosa (Mori et al. 1992; Yoshimi et al. 1995; Butterworth, Conolly, and Morgan 1995). Thus, the degree to which 1-OH-AQ and 2-OH-AQ may be mutagens is not clear from these studies.

The above studies on 1-OH-AQ and 2-OH-AQ were conducted at a time when the use of anthracene-based 1-OH-AQ and 2-OH-AQ was common and the purity of the compounds used is not clear in each case. The most probable route of manufacturing 1-OH-AQ and 2-OH-AQ was either beginning with coal tar derived AQ-OX or by a three-step process from 1- and 2-nitroanthraquinone. Both routes provided ample opportunity for mutagenic polycyclic aromatic hydrocarbon contamination.

To further illustrate this concern, in the studies reported here with 1-OH-AQ and 2-OH-AQ, a commercial source of these compounds could not be found that was not heavily contaminated (see Materials and Methods). Thus, one purpose of the current studies was to evaluate the mutagenic activity of purified 1-OH-AQ and 2-OH-AQ to bring some clarity to the issue of mutagenic potential.

9-Nitroanthracene (9-NA) at 0.12% was identified as the most prevalent contaminant in the NTP anthracene-derived AQ-OX bioassay material (Butterworth, Mathre, and Ballinger 2001). No further analytical work was done at that time. Subsequently, the question was raised as to whether the mutagenic activity of 9-NA alone was of sufficient potency to account for the degree of activity seen in the bioassay material. It was possible that the observed contaminating mutagenic and potential carcinogenic activity might reside with more than just the 9-NA. Accordingly, a precise quantitative analysis of the bioassay material was required. An NTP GC/FID (gas chromatography/flame ionization detection) analysis of the bioassay material indicated a contaminant level of 0.1% of 9-NA. However when the same material was evaluated using HPLC/UV (high-performance liquid chromatography/ultraviolet) analysis, a contaminant level of 0.5% was seen with two impurities of 0.3% and 0.2% relative to the AQ peak. The greater peak was identified as 9-NA. The second peak was not identified (NTP 2004). To resolve these discrepancies and provide needed analytical information, a more rigorous analytical evaluation of the NTP bioassay material was undertaken.

Nitroaromatic compounds can be extremely potent mutagens and carcinogens (Pitts et al. 1982; Fu et al. 1985, 1986; Durant et al. 1996). Uncertainty remained, however, regarding the biological activity of 9-NA. Was the mutagenic activity seen in the bioassay material completely accounted for by the 9-NA, or did the activity also reside with other contaminants? For example, 9-NA is only a modest bacterial mutagen whereas 2-NA is of such incredible potency to place it in the category of a super mutagen (Fu et al. 1986; NTP 2004). Only a trace amount of 2-NA would be required to contribute substantial mutagenic activity. Compounds such as 9-NA, 2-NA, and

2-nitrofluorene are active in bacterial assays without S9. Is such activity without S9 applicable for mammalian cells? Although bacterial mutagenicity is important, knowledge of genotoxic activity in mammalian cells may be more relevant. It had been reported that 9-NA was active in a mutagenicity assay in a human cell line expressing cytochrome P450 1A1 (Durant et al. 1996). To address these questions, the activity of 9-NA was evaluated in the Ames bacterial mutagenicity assay as well as the mammalian cell mouse lymphoma mutagenesis assay.

## MATERIALS AND METHODS

### Chemicals

A sample of the AQ-OX powder employed in the NTP 2-year toxicology and carcinogenesis studies (NTP 2004) was generously provided by Cynthia Smith of the NTP and Donna Browning, NTP Chemical Custodian, Battelle, Columbus, OH. This sample was designated as NTP AQ-OX. The bright yellow powder obtained from the NTP was labeled Anthraquinone, Battelle Task Identifier: 5-064-SHIP-211, lot: 5893, CAS: 84-65-1. The technical report stated that the sample had been analyzed by the NTP and was found to be about 99% anthraquinone and noted an impurity at a concentration of 0.12% (Battelle 1993; NTP 2004). Note that the AQ-OX used in the NTP companion mutagenicity studies had a contaminant level of 3% and was highly mutagenic (NTP 2004). The 9-nitroanthracene used was from Aldrich Chemical Company and was 97% pure.

### Analytical Analysis of the NTP Anthracene-Derived AQ-OX Bioassay Material

The AQ-OX preparation that was used in the NTP bioassay (NTP 2004) was studied using a rigorous analytical procedure specifically designed to quantitate impurities in AQ-OX that can be missed by conventional techniques. GC analysis of AQ-OX can often fail to detect substantial contamination with low levels of multiple contaminants because the minimum amount of material is applied to the column to avoid overloading. A more thorough analysis may be obtained if the contaminants are removed and studied separately from the main material. An extraction process based on recrystallization from a solvent compatible with HPLC analysis was evolved. The extraction solvents used were ethanol and acetonitrile. They were chosen because they have a modest solubility for the AQ, will completely dissolve the expected organic impurities, have boiling points in the range of 75°C to 85°C, have a density less than that of the solid AQ and are compatible with the mobile phase used in the HPLC analysis.

An accurately weighed sample of NTP AQ-OX between 0.030 and 0.064 g was transferred to a screwcap culture tube, 13 mm × 100 mm equipped with a Teflon-lined cap. The solvent, 5.00 ml, was added to the tube and the cap was secured. The tube was placed in a heating block such that the solvent level was about 5 to 10 mm above the top of the block. The block was heated to 75°C to 80°C to dissolve the sample and to allow the crystals of the anthraquinone to form in the zone above the top level of the

block. This zone was at a temperature below that in the lower part of the tube. Heating was continued for about 1.5 to 2 h until the original sample was completely dissolved. When the original solids had been dissolved the tube was removed from the block and placed in a beaker to cool slowly. This combination of recrystallization during the dissolution process and the slow cooling allowed for separation of a high purity anthraquinone and left the original sample impurities in the clear solution. An aliquot of this solution was diluted with HPLC mobile phase, mixed, and filtered through a 0.45-micron filter into a sample vial. The filtered solution was analyzed by HPLC using a Zorbax C18 column operated in the reverse-phase mode. A gradient elution program using acetonitrile and water was employed. The HPLC detector was run at 254 nm with data collected and processed using an electronic data system. The instrument response was calibrated with known standards.

Based on process knowledge for manufacture of AQ by oxidation of coal tar based anthracene, nitrobenzene, fluorenone, and a certified standard of polyaromatic hydrocarbons (PAHs) designated as EPA PAH 610 were purchased from Sigma Chemical (Milwaukee, WI) to provide calibration of the HPLC instrument for these compounds when present. The 9-NA standard was obtained from Fluka Chemical (Milwaukee, WI). The total method described above was validated by use of replicate samples of different weights, spikes of standard compounds added to the samples before extraction and added to the aliquots after extraction. The spike recovery for the 9-NA was consistently between 85% and 115% for spikes added to purified product at the level of 100 to 200 ppm and to sample extract at levels within the instrument calibration range. The minimum detection limit for the method was determined to be less than 2 ppm in the purified samples.

HPLC and GC detectors and configurations employed included HPLC/MS, GC/AP-MS, GC/MS, HPLC/UV, and HPLC/diode array. The HPLC/MS provided confirmation of the peaks observed in the routine HPLC/UV scans. The GC/AP-MS provided high sensitivity and specificity for determination of nitroaromatic compounds. The HPLC/diode array provided full spectra of the AQ and the 9-NA. The detection limits for the sample impurities were significantly improved to values below 5 ppm for impurities such as 9-NA, polycyclic aromatic hydrocarbons, and other impurities of interest.

### Purification of 1-OH-AQ and 2-OH-AQ for Mutagenicity Testing

No contaminant-free commercially prepared sample of either 1-OH-AQ or 2-OH-AQ could be found. For example, an HPLC examination of a commercially produced lot of 2-OH-AQ showed that that preparation contained significant levels of multiple contaminants including anthrone, 1-chloroanthraquinone, and 2-chloroanthraquinone. The contaminant level of the 1-chloroanthraquinone alone was 0.64%. The objective of these experiments was to examine the mutagenic activity of pure 1-OH-AQ and 2-OH-AQ. Therefore, obtaining pure test material was obligatory.

An attempt to purify the commercial 1-OH-AQ and 2-OH-AQ via a recrystallization process from ethanol and acetonitrile failed to reduce the impurities by more than 50%. It was concluded that use of a commercial source and standard recrystallization techniques for 1-OH-AQ and 2-OH-AQ would not yield a product of sufficient purity to be used in the bacterial mutagenicity assays. Accordingly, it was decided that the 1-OH-AQ and 2-OH-AQ would have to be synthesized, purified, and analyzed in our laboratories using techniques that would yield a pure product.

1-OH-AQ and 2-OH-AQ was synthesized starting with purified amino-anthraquinones. The purified amino-anthraquinone was converted to the hydroxyanthraquinones by the classical diazotization of the amine bisulfate. The diazo salts were hydrolyzed to the hydroxyanthraquinones with sulfuric acid. The purification of 1-OH-AQ and 2-OH-AQ was done by extraction of the product with dichloromethane to separate it from amine compounds. The dichloromethane extract was washed with water, and then extracted with 1 N sodium hydroxide in order to separate the product from impurities such as chloroanthraquinones and nitroaromatic compounds. The alkaline extract was acidified and the product was finally recrystallized from ethanol to yield the final product. HPLC analysis showed that the target impurities, amino- and chloroanthraquinones and nitroaromatic compounds, were below the method detection limits of 5 ppm.

### Bacterial Mutagenicity Assays

Samples of 1-OH-AQ, 2-OH-AQ, and 9-NA were submitted to Covance Laboratories (Vienna, VA) to test for mutagenic activity in the *Salmonella*-*Escherichia coli*/mammalian microsome reverse mutation assay. Industry accepted standard protocols and published procedures were followed in compliance with *Good Laboratory Practice* regulations (Ames, McCann, and Yamasaki 1975; Brusick et al. 1980; Maron and Ames 1983). The assay assessed the ability of the test agent to induce mutations in *Salmonella* tester strains TA98, TA100, TA1535, TA1537, and *E. coli* tester strain WP2uvrA in the presence and absence of an exogenous metabolic activation system. The activation system was a microsomal enzyme preparation derived from Aroclor-induced rat liver (S9). Dose levels were based on a toxicity range finding study. The experiments were repeated independently to confirm initial results. 9-NA was evaluated in an abbreviated screening protocol using tester strains TA98 and TA100.

Criteria for a positive response were at least a two-fold increase in the mean revertants per plate of at least one tester strain over the mean revertants per plate of the appropriate vehicle control for tester strains TA98, TA100, and WP2uvrA and/or at least a three-fold increase for tester strains TA1535 and TA1537.

### L5178Y TK+/- Mouse Lymphoma Forward Mutation Assay

A sample of 9-NA was submitted to Covance Laboratories (Vienna, VA) to test for mutagenic activity in the L5178Y TK+/-

mouse lymphoma forward mutation assay. Industry accepted standard protocols and published procedures were followed in compliance with *Good Laboratory Practice* regulations (Amacher et al. 1980; Clive et al. 1987). The objective of the assay was to evaluate the ability of 9-NA to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. DMSO was used as the vehicle. At treatment termination, precipitate was observed in the treatment medium at concentrations from 20 to 1000 µg/ml. Assays were run with and without a rat liver S9 metabolic activation system. Range-finding toxicity studies showed the testing limit to be 30 µg/ml without S9 and 50 µg/ml plus S9. The criterion for a positive response is induction of a mutation frequency that is at least two times that of the control mutant frequency for that given experiment. Colony sizing was done on cultures that produced a positive response with 9-NA.

## RESULTS

### Analytical Analysis of the NTP Anthracene-Derived AQ-OX Bioassay Material

A sample of the AQ-OX preparation that was used in the NTP bioassay (NTP 2004) was studied using a rigorous analytical procedure specifically designed to quantitate impurities in AQ-OX that can be missed by conventional techniques.

Analysis showed that the level of contamination in the bioassay material was 0.65%. The individual component in the highest amount was 9-NA at a level of 0.11%. Other classes included polycyclic aromatic hydrocarbons at 0.09% (including anthracene, phenanthrene, and dibenzo (a,h) anthracene), nitrobenzene at 0.05%, and unidentified organics and nitro-organics at 0.40%.

### Bacterial Mutagenicity of 1-OH-AQ

No commercial preparation of 1-OH-AQ was found that did not contain substantial contaminating material. Thus, 1-OH-AQ was synthesized and purified in our laboratory to meet a standard of less than 5 ppm contaminating material (see Materials and Methods).

This pure sample of 1-OH-AQ was evaluated in *Salmonella* tester strains TA98, TA100, TA1535, TA1537, and *E. coli* tester strain WP2uvrA in the presence and absence of an exogenous metabolic activation system (S9). The only activity observed for 1-OH-AQ was a weak response in TA1537 and required the presence of S9 (Table 1).

### Bacterial Mutagenicity of 2-OH-AQ

No commercial preparation of 2-OH-AQ was found that did not contain substantial contaminating material. Thus, 2-OH-AQ was synthesized and purified in our laboratory to meet a standard of less than 5 ppm contaminating material (see Materials and Methods).

**TABLE 1**  
Activity of 1-OH-AQ in the *Salmonella* and *E. coli* mutagenicity assays

1-OH-AQ $\mu\text{g}/\text{plate}$	Average revertants per plate $\pm$ SD <sup>a</sup>				
	TA98	TA100	TA1535	TA1537	WP2uvrA
Without liver microsomes (S9)					
0	14 $\pm$ 3	82 $\pm$ 12	10 $\pm$ 4	5 $\pm$ 2	14 $\pm$ 9
3.3	13 $\pm$ 5	65 $\pm$ 18	9 $\pm$ 3	10 $\pm$ 1	19 $\pm$ 4
10.0	15 $\pm$ 3	70 $\pm$ 4	12 $\pm$ 6	11 $\pm$ 2	17 $\pm$ 2
33.3	10 $\pm$ 2	80 $\pm$ 5	8 $\pm$ 3	11 $\pm$ 3	12 $\pm$ 3
100	14 $\pm$ 2	65 $\pm$ 3	11 $\pm$ 6	12 $\pm$ 5	14 $\pm$ 2
333	18 $\pm$ 4	77 $\pm$ 8	14 $\pm$ 3	8 $\pm$ 2	9 $\pm$ 3
1000	12 $\pm$ 2	71 $\pm$ 4	14 $\pm$ 2	16 $\pm$ 4	9 $\pm$ 1
Positive control <sup>b</sup>	231 $\pm$ 17 <sup>a</sup>	1114 $\pm$ 71 <sup>a</sup>	694 $\pm$ 44 <sup>a</sup>	1759 $\pm$ 157 <sup>a</sup>	141 $\pm$ 42 <sup>a</sup>
With liver microsomes (S9)					
0	27 $\pm$ 8	101 $\pm$ 14	10 $\pm$ 2	9 $\pm$ 2	15 $\pm$ 4
3.3	24 $\pm$ 6	103 $\pm$ 13	10 $\pm$ 1	45 $\pm$ 7 <sup>a</sup>	13 $\pm$ 3
10.0	25 $\pm$ 1	92 $\pm$ 11	11 $\pm$ 2	39 $\pm$ 7 <sup>a</sup>	14 $\pm$ 2
33.3	19 $\pm$ 5	103 $\pm$ 18	17 $\pm$ 4	27 $\pm$ 7 <sup>a</sup>	13 $\pm$ 3
100	26 $\pm$ 2	106 $\pm$ 1	12 $\pm$ 2	28 $\pm$ 1 <sup>a</sup>	11 $\pm$ 3
333	19 $\pm$ 5	113 $\pm$ 8	12 $\pm$ 4	18 $\pm$ 7	14 $\pm$ 2
1000	22 $\pm$ 3	102 $\pm$ 15	13 $\pm$ 3	24 $\pm$ 3	15 $\pm$ 4
Positive control <sup>c</sup>	481 $\pm$ 43 <sup>a</sup>	953 $\pm$ 273 <sup>a</sup>	144 $\pm$ 10 <sup>a</sup>	188 $\pm$ 21 <sup>a</sup>	351 $\pm$ 22 <sup>a</sup>

<sup>a</sup>Judged as a positive response. Criteria for a positive response are an increasing dose-response curve with at least one response equal to or greater than two times the mean vehicle control for tester strains TA98, TA100, and WP2uvrA and/or at least a three-fold increase for tester strains TA1535 and TA1537. A confirmatory study yielded approximately the same positive response pattern seen for TA1537 with S9.

<sup>b</sup>Positive controls without S9: TA98 1.0  $\mu\text{g}$  2-nitrofluorene; TA100 and TA1535 2.0  $\mu\text{g}$  sodium azide; TA1537 2.0  $\mu\text{g}$  ICR-191; WPuvrA 1.0  $\mu\text{g}$  4-nitroquinoline-*N*-oxide.

<sup>c</sup>Positive controls with S9: TA98 2.5  $\mu\text{g}$  benzo[a]pyrene; TA100, TA1535, and TA1537 2.5  $\mu\text{g}$  2-aminoanthracene; WpuvrA 25  $\mu\text{g}$  2-aminoanthracene.

This pure sample of 2-OH-AQ was evaluated in *Salmonella* tester strains TA98, TA100, TA1535, TA1537, and *E. coli* tester strain WP2uvrA in the presence and absence of an exogenous metabolic activation system (S9). The only activity observed for 2-OH-AQ were weak responses in strains TA100 and TA1537 and required the presence of S9 (Table 2).

### Bacterial Mutagenicity of 9-NA

9-NA was evaluated for mutagenic activity in the *Salmonella* tester strains TA98 and TA100 in the presence and absence of an exogenous metabolic activation system (S9) (Table 3). 9-NA tested positive in TA 98 minus S9 with a lowest observed effect level (LOEL) of 0.3  $\mu\text{g}/\text{plate}$  and tested positive in TA100 with a LOEL of 10  $\mu\text{g}/\text{plate}$ . No positive increases were observed in the presence of S9 at the concentrations used. The shape of the dose-response curve was not explored at higher concentrations because the compound was mutagenic at the lower doses relevant to the amount of contaminating material in the NTP cancer bioassay (Butterworth, Mathre, and Ballinger 2001).

### Mouse Lymphoma Assay with 9-NA

9-NA was evaluated for the ability to induce mutations in the L5178Y+/- mouse lymphoma forward mutation assay in the presence and absence of an S9 metabolic activation system. Range-finding studies were conducted to identify doses used in the definitive assay (see Materials and Methods). 9-NA was evaluated as negative in this assay in the absence of S9 (Table 4). 9-NA induced a positive mutagenic response in the presence of S9 beginning at doses as low as 5  $\mu\text{g}/\text{ml}$  (Table 4).

In the mouse lymphoma assay, small colony mutants carry chromosome aberrations associated with chromosome 11, the chromosome on which the TK locus is located in the mouse (Hozier et al. 1981). The positive response with 9-NA in the activation assay was primarily associated with an increase in small colonies, suggesting a predominance of a clastogenic mechanism.

### DISCUSSION

The primary purpose of the studies presented here was to provide additional information to help interpret the NTP bioassay

**TABLE 2**  
Activity of 2-OH-AQ in the *Salmonella* and *E. coli* mutagenicity assays

1-OH-AQ $\mu\text{g}/\text{plate}$	Average revertants per plate $\pm$ SD <sup>a</sup>				
	TA98	TA100	TA1535	TA1537	WP2uvrA
Without liver microsomes (S9)					
0	20 $\pm$ 6	85 $\pm$ 15	14 $\pm$ 2	9 $\pm$ 3	10 $\pm$ 0
1.0	15 $\pm$ 4	94 $\pm$ 2	13 $\pm$ 4	10 $\pm$ 1	ND
3.3	12 $\pm$ 2	88 $\pm$ 12	14 $\pm$ 4	11 $\pm$ 4	10 $\pm$ 2
10.0	16 $\pm$ 3	96 $\pm$ 7	13 $\pm$ 3	8 $\pm$ 2	9 $\pm$ 1
33.3	17 $\pm$ 3	86 $\pm$ 14	11 $\pm$ 1	8 $\pm$ 1	10 $\pm$ 3
100	12 $\pm$ 3	64 $\pm$ 5	9 $\pm$ 3	8 $\pm$ 3	8 $\pm$ 2
333	9 $\pm$ 2	10 $\pm$ 3	10 $\pm$ 2	13 $\pm$ 3	12 $\pm$ 6
1000	7 $\pm$ 2	0 $\pm$ 0	14 $\pm$ 2	13 $\pm$ 3	10 $\pm$ 5
Positive control <sup>b</sup>	204 $\pm$ 14 <sup>a</sup>	800 $\pm$ 96 <sup>a</sup>	737 $\pm$ 85 <sup>a</sup>	1150 $\pm$ 46 <sup>a</sup>	136 $\pm$ 58 <sup>a</sup>
With liver microsomes (S9)					
0	24 $\pm$ 9	94 $\pm$ 12	11 $\pm$ 3	8 $\pm$ 3	12 $\pm$ 2
0.3	28 $\pm$ 9	113 $\pm$ 26	10 $\pm$ 4	21 $\pm$ 1	ND
1.0	29 $\pm$ 6	110 $\pm$ 21	13 $\pm$ 1	37 $\pm$ 3 <sup>a</sup>	ND
3.3	24 $\pm$ 6	166 $\pm$ 13	13 $\pm$ 5	90 $\pm$ 7 <sup>a</sup>	15 $\pm$ 7
10.0	35 $\pm$ 6	195 $\pm$ 13 <sup>a</sup>	11 $\pm$ 1	114 $\pm$ 14 <sup>a</sup>	16 $\pm$ 4
33.3	29 $\pm$ 5	177 $\pm$ 11	12 $\pm$ 4	99 $\pm$ 7 <sup>a</sup>	10 $\pm$ 9
100	18 $\pm$ 2	61 $\pm$ 7	14 $\pm$ 2	53 $\pm$ 3 <sup>a</sup>	10 $\pm$ 1
333	14 $\pm$ 3	0 $\pm$ 0	8 $\pm$ 2	35 $\pm$ 13 <sup>a</sup>	10 $\pm$ 3
1000	ND	ND	ND	ND	13 $\pm$ 1
Positive control <sup>c</sup>	462 $\pm$ 28 <sup>a</sup>	1190 $\pm$ 103 <sup>a</sup>	163 $\pm$ 12 <sup>a</sup>	266 $\pm$ 62 <sup>a</sup>	466 $\pm$ 73 <sup>a</sup>

<sup>a</sup>Judged as a positive response. Criteria for a positive response are an increasing dose-response curve with at least one response equal to or greater than two times the mean vehicle control for tester strains TA98, TA100, and WP2uvrA and/or at least a three-fold increase for tester strains TA1535 and TA1537. A repeat study yielded approximately the same positive response pattern seen for TA100 and TA1537 with S9.

<sup>b</sup>Positive controls without S9: TA98 1.0  $\mu\text{g}$  2-nitrofluorene; TA100 and TA1535 2.0  $\mu\text{g}$  sodium azide; TA1537 2.0  $\mu\text{g}$  ICR-191; WPuvrA 1.0  $\mu\text{g}$  4-nitroquinoline-*N*-oxide.

<sup>c</sup>Positive controls with S9: TA98 2.5  $\mu\text{g}$  benzo[a]pyrene; TA100, TA1535, and TA1537 2.5  $\mu\text{g}$  2-aminoanthracene; WpuvrA 25  $\mu\text{g}$  2-aminoanthracene.

with anthracene-derived AQ-OX (NTP 2004). Reviewing the literature for this class of compounds showed that concerns with contamination extend to many studies with AQ and AQ derivatives. Anthracene is the starting material for AQ and several AQ derivatives. A predominant problem is that for a period of time the anthracene used was distilled from coal tar and different lots contained varying amounts of polycyclic aromatic hydrocarbon contaminants, particularly the mutagenic isomers of nitroanthracene (Cofrancesco 1992; Butterworth, Mathre, and Ballinger 2001). Butterworth, Mathre, and Ballinger (2001) identified 9-NA at 0.12% as the most prevalent contaminant in the NTP anthracene-derived AQ-OX bioassay material. The NTP reported a contaminant level in the bioassay material of 0.1% by GC analysis and of 0.5% using HPLC techniques with 9-NA as the most prevalent component. Because precise analytical information was needed in helping decide whether contaminating mutagenic and carcinogenic activity might reside

with more than just the 9-NA, a new analytical study was undertaken.

The contaminating polycyclic aromatic hydrocarbons and nitroaromatic compounds that are often present in AQ-OX can present difficult challenges for purity analysis. Conventional analytical methods often fail to detect impurities of significant concern. An improved analytical procedure was specifically developed for the NTP AQ-OX in which the contaminants were removed and studied separately from the main AQ material. After most of the AQ had been removed by recrystallization, the remaining supernatant was quantitatively analyzed for contaminants. The results of this new analysis revealed that the contamination level in the AQ bioassay material was 0.65%. The individual component in the highest amount was 9-NA at a level of 0.11%. Other classes included polycyclic aromatic hydrocarbons at 0.09% (including anthracene, phenanthrene, and dibenzo (a,h) anthracene), nitrobenzene at 0.05%,

**TABLE 3**  
Activity of 9-NA in the *Salmonella* assay

9-NA $\mu\text{g}/\text{plate}$	Revertants/plate $\pm$ SD <sup>a</sup>	
	TA98	TA100
Without liver microsomes (S9)		
0	15 $\pm$ 8	96 $\pm$ 10
0.1	25 $\pm$ 4	116 $\pm$ 1
0.3	31 $\pm$ 4 <sup>a</sup>	92 $\pm$ 8
1.0	30 $\pm$ 9 <sup>a</sup>	116 $\pm$ 6
3.0	37 $\pm$ 7 <sup>a</sup>	137 $\pm$ 4
10.0	52 $\pm$ 1 <sup>a</sup>	189 $\pm$ 8 <sup>a</sup>
Positive control <sup>b</sup>	385 $\pm$ 74 <sup>a</sup>	1348 $\pm$ 33 <sup>a</sup>
With liver microsomes (S9)		
0	24 $\pm$ 3	83 $\pm$ 18
0.1	27 $\pm$ 1	100 $\pm$ 7
0.3	31 $\pm$ 10	94 $\pm$ 1
1.0	27 $\pm$ 4	106 $\pm$ 25
3.0	32 $\pm$ 4	98 $\pm$ 28
10.0	38 $\pm$ 1	118 $\pm$ 2
Positive control <sup>c</sup>	438 $\pm$ 43 <sup>a</sup>	1397 $\pm$ 100 <sup>a</sup>

<sup>a</sup>Judged as a positive response. Criteria for a positive response are an increasing dose-response curve with at least one response equal to or greater than two times the mean vehicle control for tester strains TA98, TA100.

<sup>b</sup>Positive controls without S9: TA98 1.0  $\mu\text{g}$  2-nitrofluorene; TA100 2.0  $\mu\text{g}$  sodium azide.

<sup>c</sup>Positive controls with S9: TA98 2.5  $\mu\text{g}$  benzo[a]pyrene; TA100 2.5  $\mu\text{g}$  2-aminoanthracene.

and unidentified organics and nitro-organics at 0.40%. From the perspective of the physical amount of contaminating material, it is possible that mutagenic and carcinogenic activities in the NTP anthracene-derived AQ-OX bioassay material may reside with more contaminants than just the 9-NA.

The biological activities of 1-OH-AQ and 2-OH-AQ are relevant because they are metabolites of AQ. Blomeke et al. (1992) reported that 1-OH-AQ was positive in the Ames mutagenicity bacterial tester strain TA1537 without S9. The NTP reported that 2-OH-AQ was a potent bacterial mutagen in strain TA98 also without S9 (NTP 2004). However, neither 1-OH-AQ nor 2-OH-AQ appear to have the structure of a direct acting DNA-reactive mutagen and would not be expected to be mutagenic without metabolic activation. Once again the possibility must be addressed that such activity without S9 is actually being contributed by contaminants. Therefore, new mutagenicity studies were undertaken with purified 1-OH-AQ and 2-OH-AQ. The most revealing part of those studies was that no commercial preparation of 1-OH-AQ or 2-OH-AQ was found that did not contain substantial contaminating material (see Materials and Methods). 1-OH-AQ and 2-OH-AQ were synthesized and purified in our laboratories using procedures that lowered chloroaromatic and nitroaromatic compound levels to below 5 ppm. In the

**TABLE 4**  
Activity of 9-NA in the L5178Y mutagenicity assay

Concentration ( $\mu\text{g}/\text{ml}$ )	Relative growth (%) <sup>a</sup>	Mutant frequency ( $10\text{E}^{-6}$ units) <sup>b</sup>
9-NA minus S9		
0.0	100.0	53.7
0.1	86.8	68.0
1.0	99.6	61.7
10.0	101.0	60.7
20.0	32.7	58.3
30.0	6.4	84.1
Methylmethanesulfonate (MMS) positive control		
13 $\mu\text{g}/\text{ml}$	30.1	221.8 <sup>c</sup>
9-NA plus S9		
0.0	100.0	82.0
0.1	43.2	90.8
1.0	59.7	82.3
5.0	25.0	176.4 <sup>d</sup>
10.0	14.5	198.8 <sup>d</sup>
50.0	5.3	321.8 <sup>d</sup>
Methylcholanthrene (MCA) positive control		
2 $\mu\text{g}/\text{ml}$	51.2	284.7 <sup>d</sup>

<sup>a</sup>Relative growth = (relative suspension growth  $\times$  relative cloning efficiency)/100.

<sup>b</sup>Mutant frequency = (total mutant colonies/total viable colonies)  $\times 2 \times 10\text{E}^{-4}$ . Decimal is moved to express the frequency in units of  $10\text{E}^{-6}$ .

<sup>c</sup>Mutagenic as judged by exceeding the minimum criterion of  $10^7$  for this experiment (two times the control frequency).

<sup>d</sup>Mutagenic as judged by exceeding the minimum criterion of 164 for this experiment (two times the control frequency).

bacterial mutagenicity assays, the only activity observed for 1-OH-AQ was a weak response in TA1537 and required the presence of S9 (Table 1). The only activity observed for 2-OH-AQ were weak responses in TA100 and TA1537 and required the presence of S9 (Table 2).

These observations suggest that the activity reported for 1-OH-AQ and 2-OH-AQ without S9 are due to contaminants (Blomeke et al. 1992; NTP 2004). Thus, the comparative mutagenic potency calculations used by the NTP for these compounds are likely not valid (NTP 2004). It is not known why weak responses with S9 were seen with the 1-OH-AQ and 2-OH-AQ metabolites of AQ, whereas numerous genetic toxicology assays (all of which included metabolic activation) showed no activity at all with the parent compound AQ. It would appear that the amounts produced by metabolism and the resulting activity is simply too small to register in those assays with AQ.

Many nitroaromatic compounds are extremely potent mutagens and carcinogens (Pitts et al. 1982; Fu et al. 1985, 1986; Durant et al. 1996). Thus, the finding that 9-NA was a primary contaminant in the NTP anthracene-derived AQ-OX bioassay material raised several issues related to this class of compound.

9-NA tested positive in TA98 without S9 with a LOEL of 0.3  $\mu\text{g}/\text{plate}$  and tested positive in TA100 with a LOEL of 10  $\mu\text{g}/\text{plate}$  (Table 3). This pattern of activity without S9 is consistent with that seen with the anthracene-derived AQ-OX used in the NTP bioassay and suggests that 9-NA was responsible for at least part of the observed mutagenic activity. The low LOEL values observed are one indicator of potent mutagenic activity. The magnitude of the response, however, was not sufficient to account for all of the mutagenic response seen with the bioassay material (Butterworth, Mathre, and Ballinger 2001). Therefore, the mutagenic activity noted in the bioassay material would appear to reside with more than just the 9-NA. This is consistent with the results of the analytical study presented above.

One interesting property of many of the nitroaromatic compounds is that they exhibit activity in bacterial mutagenesis assays without added metabolic activation. Examples of compounds with such activity include 9-NA, 2-NA, and 2-nitrofluorene. The latter chemical is often used as a positive control in the Ames test without S9. These compounds, therefore, appear to in some way be either directly DNA reactive or are metabolized by the tester bacteria to reactive metabolites. Caution must be used in equating such activity in bacteria to activity in mammalian cells. Knowledge of activity in mammalian cells is more relevant in interpreting the potential influence of 9-NA on the NTP cancer bioassay. 9-NA exhibits mutagenic activity in a human cell line expressing cytochrome P450 1A1 (Durant et al. 1996). In contrast to the bacterial assays, 9-NA was evaluated as negative in the mammalian cell mouse lymphoma mutagenicity assay in the absence of S9 (Table 4). However, 9-NA induced a positive mutagenic response in the presence of S9 beginning at doses as low as 5  $\mu\text{g}/\text{ml}$  (Table 4). The positive response with 9-NA in the activation assay was primarily associated with an increase in small colonies suggesting a predominance of a clastogenic mechanism. This is particularly relevant because clastogens are frequently potent carcinogens.

Although the NTP bioassay is directly applicable to exposure to anthracene-derived AQ-OX, the degree to which the contaminants may have influenced the induction of tumors is not known. Based on extensive testing of purified materials, AQ not a genotoxic carcinogen. Quantitative bacterial mutagenicity and carcinogenicity potency estimates indicate that it is plausible that the contaminants alone could have been responsible for all of the observed carcinogenic activity (Butterworth, Mathre, and Ballinger 2001). The finding that the primary contaminant, 9-NA, is a potent mammalian cell mutagen and clastogen increases the possibility that the contaminants, rather than AQ, produced the carcinogenic response.

These concerns have not been lost on those working in this area. The NTP recognized and addressed the contamination issue in their report (NTP 2004). Because of the numerous toxicology studies related to this issue, the coal tar based anthracene-derived AQ-OX production process is now seldom used. Industry pays a great deal of attention to sources and purity of AQ and AQ-OX is no longer commercially used in the United States. Analyt-

ical purity and verification are now more regular components of genetic toxicology testing protocols. The lingering legacy is, however, that many of the reported results in the literature for AQ and AQ derivative compounds need to be viewed with caution.

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## The preparation of anthraquinone used in the National Toxicology Program cancer bioassay was contaminated with the mutagen 9-nitroanthracene

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Commercial anthraquinone (AQ) (9,10-anthracenedione) is produced by at least three different production methods worldwide: oxidation of anthracene (AQ-OX), Friedel–Crafts technology (AQ-FC) and by Diels–Alder chemistry (AQ-DA), with the final product varying in color and purity. AQ-OX begins with anthracene produced from coal tar and different lots can contain various contaminants, particularly the mutagenic isomers of nitroanthracene. AQ has been reported to be negative in a variety of genotoxicity tests including numerous Ames *Salmonella* mutagenicity assays. In addition, we report that AQ-DA is negative in the *Salmonella–Escherichia coli* reverse mutation assays, the L5178Y mouse lymphoma forward mutation assay, for inducing chromosomal aberrations, polyploidy or endoreduplication in Chinese hamster ovary cells, and in the *in vivo* mouse micronucleus assay. Further, a previous 18 month bioassay conducted with AQ administered to male and female B6C3F<sub>1</sub> and (C57BL/6 × AKR)F<sub>1</sub> mice reported no induction of cancer. Thus, it was somewhat unexpected that in a long-term study conducted by the National Toxicology Program (NTP) AQ-OX induced a weak to modest increase in tumors in the kidney and bladder of male and female F344/N rats and a strong increase in the livers of male and female B6C3F<sub>1</sub> mice. In the studies reported here, a sample of the AQ-OX used in the NTP bioassay was shown to be mutagenic in the Ames tester strains TA98, TA100 and TA1537. Addition of an S9 metabolic activation system decreased or eliminated the mutagenic activity. In contrast, the purified NTP AQ-OX as well as the technical grade samples AQ-FC and AQ-DA were not mutagenic in the Ames test. The chemical structure of AQ does not suggest that the parent compound would be DNA reactive. Therefore, a mutagenic contaminant was present in the NTP bioassay sample that is either directly mutagenic or can be activated by bacterial metabolism. Analytical studies showed that the primary contaminant 9-nitroanthracene (9-NA) was present in the NTP AQ-OX at a concentration of 1200 p.p.m., but not in the purified material. The 9-NA and any other contaminants that might have been present in the NTP AQ-OX induced measurable mutagenicity at 9-NA concentrations as low as 0.15 µg/plate in tester strain TA98, indicating potent mutagenic activity. On the basis of revertants per microgram, 9-NA was more potent than benzo[*a*]pyrene (B[*a*]P) and was about equally as potent as the 2-nitrofluorene run concurrently as positive controls. TD<sub>50</sub> quantitative carcinogenicity potency estimates indicate that a carcinogen of a potency

in the range between B[*a*]P and dimethylnitrosamine would be required to produce the observed carcinogenic response at the levels of the contaminants found in the test sample. While recognizing that there are limitations in extrapolating mutagenic potency to potential carcinogenic potency, these estimates do indicate that it is plausible that the 9-NA contaminant might have been responsible for all of the tumor induction observed in the NTP study. In fact, in the absence of reliable cancer data, the genetic toxicology profile indicates that AQ would not be a genotoxic carcinogen. Thus, no conclusion as to the carcinogenic activity of AQ can be made at this time.

### Introduction

#### *The importance of source and purity*

Anthraquinone (AQ) (9,10-anthracenedione) is used to enhance the efficiency of the Kraft Process for the production of paper, thus reducing the demand for trees to be cut down (Cofrancesco, 1992). AQ is the active ingredient in the most effective and non-harmful bird repellent used, for example, for keeping birds from airport runways or areas where they would conflict with the human population (Ballinger and Price, 1996; Cummings *et al.*, 1997; Ballinger *et al.*, 1998; Dolbeer *et al.*, 1998).

In assessing the potential biological activity of preparations of AQ, it is critical to be aware of how the preparation of interest was manufactured and the potential contaminants inherent with the different synthesis processes. AQ is produced in large quantities by at least three different production methods in various parts of the world (Cofrancesco, 1992). The oxidation of anthracene to yield AQ is the oldest known production process and is now practiced primarily in Europe. AQ from the oxidation process (AQ-OX) involves the oxidation of anthracene derived from coal tar. The quality of the AQ-OX produced is dependent on the number of contaminating high-boiling mutagenic and carcinogenic polycyclic aromatic hydrocarbons (PAHs), which co-distill with anthracene, found in the starting material. Profiles of contaminants from this process can differ substantially. Of particular concern is the observation that the mutagenic nitroanthracenes are often seen in AQ-OX preparations, sometimes at concentrations >2500 p.p.m. (US EPA, 1977; ICI, 1978a, 1978b).

Benzene and phthalic anhydride undergo the Friedel–Crafts reaction to yield *o*-benzoylbenzoic acid, which is treated with concentrated sulfuric acid to yield AQ. This is the most prevalent production method employed in China and India. AQ produced by the Friedel–Crafts process (AQ-FC) is substantially free of the PAH contaminants and nitroanthracenes that can be found in AQ-OX.

Production of AQ by the Diels–Alder reaction (AQ-DA) between 1,4-naphthoquinone and 1,3-butadiene is practiced primarily in Japan. Because this process involves shifts between

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the aqueous and organic phases, contaminants are easily removed and AQ-DA is particularly clean and free of contaminants.

To our knowledge, all AQ used commercially in the US is either AQ-FC or AQ-DA, rather than AQ-OX. Interestingly, the reagent grade material supplied to research laboratories is often AQ-OX. The National Toxicology Program (NTP) recently completed a cancer bioassay with AQ showing that it exhibited weak to modest carcinogenic activity (NTP, 1999). The material employed in that bioassay was from the oxidation process, AQ-OX, and contained an unidentified peak by GC analysis at a level of 0.12% (Battelle, 1993; NTP, 1999). One purpose of the studies presented here was to identify and quantify that and other potential contaminants in the material used by the NTP.

#### Genotoxicity

A large number of mutagenicity assays have reported that neither AQ nor its metabolites exhibit genotoxic activity. Negative results in the Ames *Salmonella* bacterial mutagenicity assay have been reported by seven independent laboratories (Brown and Brown, 1976; Anderson and Stiles, 1978; Gibson *et al.*, 1978; Salamone *et al.*, 1979; Sakai *et al.*, 1985; Tikkanen *et al.*, 1983; National Cancer Institute, 1987). AQ is negative in the Syrian hamster embryo (SHE) cell transformation assay (Kerckaert *et al.*, 1996). AQ is also not mutagenic in a line of human B-lymphoblastoid cells that constitutively express cytochrome P4501A1 (Durant *et al.*, 1996). An 18 month bioassay conducted with AQ administered to male and female B6C3F<sub>1</sub> and (C57BL/6×AKR)F<sub>1</sub> mice reported no induction of cancer (Innes *et al.*, 1969). In that study, AQ composition was confirmed by infrared spectroscopy, gas chromatography and thin-layer chromatography. No contaminants were reported (Innes *et al.*, 1969).

Thus, it was somewhat unexpected when the NTP reported that AQ induced a weak to modest increase in tumors in the kidney and bladder of male and female F344/N rats and a strong increase in the livers of male and female B6C3F<sub>1</sub> mice (NTP, 1999). In contrast to the numerous papers documenting a lack of AQ mutagenic activity noted above, two papers reported that AQ was mutagenic in the Ames *Salmonella* mutagenicity assay. The pattern of activity of AQ reported was, however, unusual in that mutagenic activity was seen without metabolic activation, and that addition of an S9 metabolic activation system reduced or eliminated the response (Lieberman *et al.*, 1982; Zeiger *et al.*, 1988). The chemical structure of AQ does not suggest that the parent compound would be a DNA reactive mutagen. Therefore, it appeared that a mutagenic contaminant was present in the positive Ames test samples that was either directly mutagenic or could be activated by bacterial metabolism. Similarly, there is one report of weak induction of micronuclei in SHE cells, but the material used was the NTP AQ-OX (Gibson *et al.*, 1997) and, as noted below, several other micronuclei assays are negative.

In fact, the problem of contamination of AQ-OX with nitroanthracenes producing a mutagenic preparation has been well documented (US EPA, 1977; ICI, 1978a, 1978b). For example, for a TSCA submission, six samples of AQ were submitted to the Ames *Salmonella* mutagenicity assay (US EPA, 1977). Only one was positive, and that was without metabolic activation. It was concluded that the mutagenic activity came from contamination of the sample with 9-nitroanthracene (9-NA). When that sample was purified and retested, it showed no mutagenic activity.

**Table I.** Contaminants in preparations of AQ

<b>NTP AQ-OX</b>	
Component	Concentration <sup>a</sup>
AQ	99%
9-NA	1200 p.p.m. <sup>b</sup>
phenanthrene	200 p.p.m.
target toxic compounds <sup>c</sup>	ND <sup>d</sup>
<b>Purified NTP AQ-OX</b>	
Component	Concentration <sup>a</sup>
AQ	99%
9-NA	ND <sup>e</sup>
phenanthrene	ND <sup>d</sup>
target toxic compounds <sup>c</sup>	ND <sup>d</sup>
<b>AQ-FC</b>	
Component	Concentration <sup>a</sup>
AQ	99%
9-NA	ND <sup>e</sup>
target toxic compounds <sup>c</sup>	ND <sup>d</sup>
<b>AQ-DA</b>	
Component	Concentration <sup>a</sup>
AQ	99%
9-NA	ND <sup>e</sup>
target toxic compounds <sup>c</sup>	ND <sup>d</sup>

<sup>a</sup>GC-MS analyses were conducted to identify impurities using the conditions specified in US EPA Methods 610, 625 and 8270 in the Code of Federal Register 40. Quantitation of contaminants was based on HPLC analysis.

<sup>b</sup>Noted in the NTP report at 0.12% but not identified at that time (NTP, 1999).

<sup>c</sup>US EPA Methods 610, 625 and 8270 in the Code of Federal Register 40 identify 16 PAH and 72 additional toxic or carcinogenic substances of concern in environmental samples. Analyses were done using the conditions and looking for the target toxic compounds specified in these standard procedures.

<sup>d</sup>Not detected (ND) at a limit of detection of <50 p.p.m.

<sup>e</sup>Not detected (ND) at a limit of detection of <5 p.p.m.

It is clear that samples of technical grade AQ can vary widely in how they are produced, and the types and amounts of impurities. Another purpose of these studies was to determine the mutagenic activity of the actual material used in the NTP bioassay and to contrast that with other preparations of AQ. A sample of the archived NTP AQ-OX was generously provided by the NTP. The activity of this material was contrasted in the Ames *Salmonella* mutagenicity assay with purified NTP AQ-OX as well as samples of AQ-FC and AQ-DA. In addition, results from several additional genotoxicity assays are reported using the AQ-DA material.

## Materials and methods

### Chemicals

A sample of the AQ-OX powder employed in the NTP 2-year toxicology and carcinogenesis studies (NTP, 1999) was generously provided by Cynthia Smith of the NTP and Donna Browning, NTP Chemical Custodian (Battelle, Columbus, OH). This sample was designated as NTP AQ-OX. The bright yellow powder obtained from the NTP was labeled Anthraquinone, Battelle Task Identifier: 5-064-SHIP-211, lot: 5893, CAS: 84-65-1. The technical report stated that the sample had been analyzed by the NTP and was found to be ~99% AQ and noted an impurity at a concentration of 0.12% (Battelle, 1993; NTP, 1999).

A portion of the NTP AQ-OX was purified by 2× recrystallization from ethanol. This sample was designated as purified NTP AQ-OX.

A sample of technical grade AQ-FC typical of that in commercial use was obtained from Environmental Biocontrol, Intl. (Wilmington, DE). This sample was designated as AQ-FC.

Samples of technical grade AQ-DA typical of that in commercial use were obtained from Environmental Biocontrol, Intl. (Wilmington, DE). These were used over a period of several months for the various genotoxicity assays

**Table II.** Activity of NTP AQ-OX in the Ames and *E. coli* mutagenicity assays

Without liver microsomes (S9) AQ-OX µg/plate	9-NA <sup>a</sup> µg/plate	Average revertants per plate ± SD <sup>a</sup>				
		TA98	TA100	TA1535	TA1537	WP2uvrA
0	0	18 ± 4	89 ± 9	12 ± 2	7 ± 3	15 ± 4
30	0.04	20 ± 3	107 ± 14	12 ± 6	10 ± 1	14 ± 2
60	0.07	25 ± 10	113 ± 15	12 ± 1	10 ± 3	13 ± 5
125	0.15	42 ± 6 <sup>b</sup>	113 ± 18	10 ± 4	8 ± 4	10 ± 3
250	0.3	62 ± 5 <sup>b</sup>	127 ± 18	16 ± 7	21 ± 10 <sup>b</sup>	12 ± 6
500	0.6	116 ± 16 <sup>b</sup>	142 ± 4	17 ± 9	26 ± 5 <sup>b</sup>	20 ± 1
1000	1.2	213 ± 29 <sup>b</sup>	131 ± 21	11 ± 1	40 ± 5 <sup>b</sup>	10 ± 2
2000	2.4	433 ± 40 <sup>b</sup>	220 ± 6 <sup>b</sup>	18 ± 3	95 ± 6 <sup>b</sup>	16 ± 6
pos. con.		193 ± 23 <sup>c</sup>	617 ± 6 <sup>c</sup>	589 ± 96 <sup>c</sup>	503 ± 21 <sup>c</sup>	145 ± 6 <sup>c</sup>
With liver microsomes (S9)						
0	0	30 ± 8	149 ± 4	20 ± 1	10 ± 4	15 ± 3
30	0.04	33 ± 4	140 ± 19	19 ± 3	12 ± 2	14 ± 3
60	0.07	30 ± 9	138 ± 15	25 ± 4	11 ± 3	14 ± 1
125	0.15	32 ± 4	134 ± 3	19 ± 1	14 ± 1	20 ± 5
250	0.3	37 ± 4	127 ± 2	20 ± 4	11 ± 4	12 ± 5
500	0.6	52 ± 5	130 ± 9	18 ± 5	18 ± 2	12 ± 4
1000	1.2	102 ± 11	147 ± 21	20 ± 6	20 ± 4	16 ± 4
2000	2.4	162 ± 13	164 ± 22	21 ± 1	32 ± 8	15 ± 4
pos. con.		373 ± 17 <sup>d</sup>	534 ± 81 <sup>d</sup>	78 ± 5 <sup>d</sup>	111 ± 12 <sup>d</sup>	245 ± 21 <sup>d</sup>

<sup>a</sup>NTP AQ-OX contains 9-NA at a level of 1200 p.p.m. This column shows the calculated amount of this contaminant on the plate. Since AQ is not mutagenic, the mutagenic activity can be ascribed to the 9-NA and any other contaminants that might have been present. Since 9-NA was the major contaminant identified with analytical studies, and as a first approximation, mutagenic activity has been assumed to arise from the 9-NA. Nevertheless, the same issues and conclusions hold even if mutagenic activity was to be shown to arise from several different contaminants.

<sup>b</sup>Judged as a positive response. Criteria for a positive response are an increasing dose-response curve with at least one response equal to or greater than twice the mean vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least three times the tester strains TA1535 and TA1537.

<sup>c</sup>Positive controls without S9: TA98 1.0 µg 2-nitrofluorene; TA100 and TA1535 2.0 µg sodium azide; TA1537 2.0 µg ICR-191; WPuvrA 1.0 µg 4-nitroquinoline-*N*-oxide.

<sup>d</sup>Positive controls with S9: TA98 2.5 µg B[a]P; TA100, TA1535 and TA1537 2.5 µg 2-aminoanthracene; WpuvrA 25 µg 2-aminoanthracene.

presented here. High quality control standards and analytical verification indicated that these samples were very uniform. These samples were designated as AQ-DA.

Analytical techniques including high-performance liquid chromatography (HPLC) and GC-MS were performed by DCV Group (Wilmington, DE) and Covance Laboratories (Leesburg, VA) to verify that the main component in all preparations was AQ, that purification did not alter the parent compound and to identify potential contaminants (Table I). GC-MS analysis was conducted to identify impurities. US EPA Methods 610, 625 and 8270 in the Code of Federal Register 40 identify 16 PAH and 72 additional toxic or carcinogenic substances of concern in environmental samples. Analyses were done using the conditions and looking for the target toxic compounds specified in these standard procedures. Quantitation of contaminants was based on HPLC analysis.

#### Bacterial mutagenicity assays

Samples NTP AQ-OX, purified NTP AQ-OX, AQ-FC and AQ-DA were submitted to Covance Laboratories (Vienna, VA) to test for mutagenic activity in the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a confirmatory assay. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Ames *et al.*, 1975; Brusick *et al.*, 1980; Maron and Ames; 1983). The assay tested for the ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains (Ames test), and the tryptophan locus in an *E. coli* tester strain both in the presence and absence of an exogenous metabolic activation system (Ames *et al.*, 1975; Brusick *et al.*, 1980; Maron and Ames; 1983). The activation system was a microsomal enzyme preparation derived from Aroclor-induced rat liver (S9). Dose levels were based on a toxicity range-finding study. The top doses demonstrated test article precipitate on the plates. No appreciable toxicity was observed with any of the samples. The experiments were repeated independently to confirm initial results.

Criteria for a positive response were at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean revertants per plate of the appropriate vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least a 3-fold increase for tester strains TA1535 and TA1537.

#### L5178Y thymidine kinase (TK)+/- mouse lymphoma forward mutation assay

A sample of AQ-DA was submitted to Covance Laboratories (Vienna, VA) to test for mutagenic activity in the L5178Y TK+/- mouse lymphoma forward mutation assay. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Amacher *et al.*, 1980; Clive *et al.*, 1987). The objective of the assay was to evaluate the ability of AQ to induce forward mutations at the TK locus in the mouse lymphoma L5178Y cell line. The test article formed a suspension in dimethylsulfoxide at concentrations >1.56 mg/kg and was insoluble in medium above ~25 µg/ml. Assays were run with and without a rat liver S9 metabolic activation system. Range-finding studies showed AQ to be non-toxic at nominal doses up to 500 µg/ml with and without metabolic activation. The testing limit for the mutation assays was set at 50 µg/ml, which is about twice the solubility limit in medium. Two independent assays were conducted each with and without metabolic activation. The criterion for a positive response is induction of a mutation frequency that is at least two times that of the control mutant frequency for that given experiment. Colony sizing was not done because no positive responses were observed with AQ.

#### Chromosomal aberration induction in Chinese hamster ovary (CHO) cells

A sample of AQ-DA was submitted to Covance Laboratories (Vienna, VA) to test for the ability to induce chromosomal aberrations in CHO cells with and without metabolic activation. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Evans, 1962). A dose range-finding study was conducted to select experimental doses. Solubility considerations determined the highest dose to be 50 µg/ml. Replicate cultures of CHO cells were incubated with up to 50 µg/ml AQ-DA with and without metabolic activation, with a 20.0 h harvest in an initial trial and with 20.0 and 44.0 harvests in the confirmatory trials. No visual signs of cytotoxicity were observed in the cultures analyzed. A test article was considered positive for inducing chromosomal aberrations if a significant increase was observed compared to controls at a level of  $P < 0.01$

#### In vivo bone marrow mouse micronucleus assay

A sample of AQ-DA was submitted to Covance Laboratories (Vienna, VA) for evaluation in the *in vivo* mouse micronucleus assay. The objective of this whole animal assay was to evaluate the ability of AQ-DA to induce micronuclei

**Table III.** Activity of purified NTP AQ-OX in the Ames and *E.coli* mutagenicity assays

Without liver microsomes (S9) Purified AQ-OX µg/plate	Average revertants per plate ± SD				
	TA98	TA100	TA1535	TA1537	WP2uvrA
0	14 ± 4	75 ± 4	10 ± 4	6 ± 2	15 ± 5
30	16 ± 3	87 ± 8	15 ± 7	9 ± 1	18 ± 4
60	20 ± 1	81 ± 11	6 ± 5	6 ± 3	15 ± 4
125	15 ± 4	80 ± 4	8 ± 3	8 ± 4	13 ± 3
250	10 ± 3	83 ± 12	9 ± 1	5 ± 4	10 ± 2
500	11 ± 2	80 ± 9	13 ± 4	6 ± 1	12 ± 1
1000	15 ± 2	95 ± 2	13 ± 3	7 ± 2	14 ± 5
2000	22 ± 3	86 ± 8	13 ± 5	6 ± 1	13 ± 3
pos. con.	121 ± 10 <sup>b</sup>	533 ± 8 <sup>b</sup>	418 ± 17 <sup>b</sup>	657 ± 38 <sup>b</sup>	132 ± 26 <sup>b</sup>
With liver microsomes (S9)					
0	21 ± 3	93 ± 15	13 ± 1	8 ± 2	14 ± 3
30	23 ± 3	96 ± 14	13 ± 6	10 ± 5	18 ± 2
60	32 ± 3	88 ± 17	8 ± 2	10 ± 3	20 ± 5
125	31 ± 6	84 ± 10	11 ± 1	9 ± 2	19 ± 2
250	26 ± 5	75 ± 14	10 ± 4	9 ± 1	18 ± 4
500	28 ± 5	79 ± 6	6 ± 0	9 ± 3	19 ± 4
1000	28 ± 2	87 ± 10	9 ± 2	9 ± 5	17 ± 2
2000	29 ± 3	86 ± 7	14 ± 4	7 ± 2	22 ± 6
pos. con.	321 ± 18 <sup>c</sup>	582 ± 22 <sup>c</sup>	135 ± 12 <sup>c</sup>	565 ± 105 <sup>c</sup>	350 ± 23 <sup>c</sup>

<sup>a</sup>Criteria for a positive response are an increasing dose-response curve with at least one response equal to or greater than twice the mean vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least three times the tester strains TA1535 and TA1537.

<sup>b</sup>Positive controls without S9: TA98 1.0 µg 2-nitrofluorene; TA100 and TA1535 2.0 µg sodium azide; TA1537 2.0 µg ICR-191; WPuvrA 1.0 µg 4-nitroquinoline-*N*-oxide.

<sup>c</sup>Positive controls with S9: TA98 2.5 µg B[a]P; TA100, TA1535 and TA1537 2.5 µg 2-aminoanthracene; WpuvrA 25 µg 2-aminoanthracene.

**Table IV.** Activity of AQ-FC in the Ames and *E.coli* mutagenicity assays

Without liver microsomes (S9) Purified AQ-FC µg/plate	Average revertants per plate ± SD <sup>a</sup>				
	TA98	TA100	TA1535	TA1537	WP2uvrA
0	14 ± 3	93 ± 9	13 ± 3	9 ± 1	18 ± 2
30	11 ± 4	89 ± 6	13 ± 1	6 ± 3	17 ± 6
60	12 ± 3	92 ± 11	9 ± 3	5 ± 2	19 ± 6
125	14 ± 3	94 ± 11	10 ± 3	5 ± 3	16 ± 3
250	17 ± 6	93 ± 8	12 ± 6	6 ± 1	17 ± 8
500	16 ± 5	107 ± 7	10 ± 1	9 ± 5	13 ± 3
1000	14 ± 6	96 ± 12	10 ± 2	7 ± 2	17 ± 2
2000	14 ± 4	98 ± 5	12 ± 2	6 ± 4	14 ± 6
pos. con.	156 ± 8 <sup>b</sup>	547 ± 16 <sup>b</sup>	482 ± 10 <sup>b</sup>	421 ± 33 <sup>b</sup>	152 ± 19 <sup>b</sup>
With liver microsomes (S9)					
0	24 ± 6	123 ± 13	10 ± 6	5 ± 2	18 ± 4
30	16 ± 5	91 ± 8	9 ± 2	6 ± 4	18 ± 3
60	23 ± 9	95 ± 12	12 ± 1	8 ± 2	20 ± 3
125	19 ± 9	92 ± 8	14 ± 2	8 ± 3	18 ± 3
250	20 ± 5	107 ± 10	10 ± 4	7 ± 1	20 ± 2
500	19 ± 3	104 ± 5	10 ± 2	8 ± 3	21 ± 4
1000	22 ± 3	93 ± 7	12 ± 6	8 ± 4	18 ± 3
2000	21 ± 0	116 ± 10	12 ± 7	8 ± 2	20 ± 6
pos. con.	351 ± 20 <sup>c</sup>	916 ± 20 <sup>c</sup>	138 ± 10 <sup>c</sup>	143 ± 9 <sup>c</sup>	355 ± 41 <sup>c</sup>

<sup>a</sup>Criteria for a positive response are an increasing dose-response curve with at least one response equal to or greater than twice the mean vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least three times the tester strains TA1535 and TA1537.

<sup>b</sup>Positive controls without S9: TA98 1.0 µg 2-nitrofluorene; TA100 and TA1535 2.0 µg sodium azide; TA1537 2.0 µg ICR-191; WPuvrA 1.0 µg 4-nitroquinoline-*N*-oxide.

<sup>c</sup>Positive controls with S9: TA98 2.5 µg B[a]P; TA100, TA1535 and TA1537 2.5 µg 2-aminoanthracene; WpuvrA 25 µg 2-aminoanthracene.

in bone marrow polychromatic erythrocytes (PCE) of CrI:CD-1 (ICR) BR mice. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Heddle *et al.*, 1991; Salamone and Mavournin, 1994; Schmid, 1976). In the dose selection study the test article was suspended in corn oil and dosed by oral

gavage at up to 5000 mg/kg. Based on lack of significant toxicity, 5000 mg/kg was selected as the highest dose for the micronucleus studies. In the micronucleus assay, AQ-DA was suspended in corn oil and dosed by oral gavage at 1250, 2500 and 5000 mg/kg. Five males and five females were randomly assigned to each dose per harvest time group. Animals were killed

**Table V.** Activity of AQ-DA in the Ames and *E.coli* mutagenicity assays

Without liver microsomes (S9) Purified AQ-FC µg/plate	Average revertants per plate ± SD <sup>a</sup>				
	TA98	TA100	TA1535	TA1537	WP2uvrA
0	13 ± 5	96 ± 7	9 ± 3	7 ± 1	18 ± 2
30	15 ± 1	92 ± 6	11 ± 4	6 ± 1	17 ± 6
60	15 ± 3	93 ± 12	13 ± 1	11 ± 2	17 ± 2
125	13 ± 2	89 ± 8	11 ± 2	4 ± 3	18 ± 1
250	16 ± 3	94 ± 9	10 ± 2	7 ± 1	18 ± 2
500	12 ± 4	96 ± 13	9 ± 4	5 ± 1	19 ± 8
1000	15 ± 3	96 ± 4	11 ± 0	5 ± 2	18 ± 2
2000	14 ± 2	105 ± 8	11 ± 1	8 ± 2	20 ± 4
pos. con.	188 ± 9 <sup>b</sup>	498 ± 65 <sup>b</sup>	444 ± 31 <sup>b</sup>	612 ± 83 <sup>b</sup>	119 ± 14 <sup>b</sup>
With liver microsomes (S9)					
0	13 ± 2	72 ± 4	9 ± 4	9 ± 1	20 ± 3
30	12 ± 5	73 ± 6	10 ± 2	9 ± 1	23 ± 3
60	12 ± 6	77 ± 8	6 ± 1	9 ± 1	19 ± 6
125	14 ± 5	85 ± 13	12 ± 2	13 ± 2	22 ± 8
250	11 ± 2	83 ± 7	12 ± 1	9 ± 3	19 ± 1
500	15 ± 7	77 ± 9	9 ± 6	8 ± 1	18 ± 4
1000	9 ± 1	82 ± 5	10 ± 4	9 ± 2	19 ± 6
2000	9 ± 1	82 ± 5	10 ± 4	11 ± 4	16 ± 8
pos. con.	191 ± 21 <sup>c</sup>	582 ± 22 <sup>c</sup>	519 ± 31 <sup>c</sup>	126 ± 25 <sup>c</sup>	301 ± 12 <sup>c</sup>

<sup>a</sup>Criteria for a positive response are an increasing dose–response curve with at least one response equal to or greater than twice the mean vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least three times the tester strains TA1535 and TA1537.

<sup>b</sup>Positive controls without S9: TA98 1.0 µg 2-nitrofluorene; TA100 and TA1535 2.0 µg sodium azide; TA1537 2.0 µg ICR-191; WPuvrA 1.0 µg 4-nitroquinoline-*N*-oxide.

<sup>c</sup>Positive controls with S9: TA98 2.5 µg B[a]P; TA100, TA1535 and TA1537 2.5 µg 2-aminoanthracene; WpuvrA 25 µg 2-aminoanthracene.

~24, 48 and 72 h after dosing with the test article for extraction of the bone marrow. A response was judged positive if it was significantly greater than the corresponding vehicle control at a level of  $P < 0.01$ .

## Results

### Quantitative analysis

In every case the main component of the test material was confirmed to be AQ (Table I). Thus, purification of the NTP AQ-OX did not alter the main AQ constituent. The NTP report on AQ (Battelle, 1993; NTP, 1999) noted an extra peak on the GC trace in the AQ-OX at a level of 1200 p.p.m., but did not identify the contaminant at that time. The same peak was observed also at 1200 p.p.m. in the analytical studies reported here and was identified as 9-NA. The purification procedure removed the 9-NA from the NTP AQ-OX (Table I).

### Bacterial mutation assays

All samples were run in the same laboratory under identical conditions. The NTP AQ-OX sample was mutagenic in a dose-dependent manner in strains TA98, TA100 and TA1537 (Table II). Mutagenic activity was reduced or eliminated by addition of an S9 rat liver microsomal metabolic activation system. In contrast, no mutagenic activity was observed with the purified NTP AQ-OX (Table III). Because AQ is not mutagenic, all the mutagenic activity in the NTP AQ-OX sample can be ascribed to the 9-NA. Mutagenic activity of the 9-NA is seen at concentrations as low as 0.15 µg/plate, indicating potent activity (Table II). In tester strain TA98, the induced revertants over controls per microgram of chemical for the concurrently run positive controls are 137 revertants/µg for benzo[*a*]pyrene (B[a]P), and 175 revertants/µg for 2-nitrofluorene. The 9-NA with 173 revertants/µg is more potent than B[a]P and equal in potency to 2-nitrofluorene (Table II).

Neither AQ-FC nor AQ-DA showed any mutagenic activity

**Table VI.** Activity of AQ-DA in the L5178Y mutagenicity assay

Concentration	Relative growth (%) <sup>a</sup>	Mutant frequency (10 <sup>-6</sup> U) <sup>b</sup>
Technical 1 AQ-DA without metabolic activation (µg/ml)		
0.0	100.0	82.6
3.13	93.4	72.1
6.25	78.6	82.8
12.5	99.3	71.0
25.0	74.8	85.9
37.5	75.2	79.5
50.0	81.1	83.1
Methylmethanesulfonate positive control		
5 nl/ml	7.4	923.9 <sup>c</sup>
Technical 1 AQ-DA with metabolic activation (µg/ml)		
0.0	100.0	114.3
1.57	108.7	180.5
3.13	101.2	158.6
6.25	85.9	139.8
12.50	104.6	129.5
25.0	122.1	127.0
37.5	97.9	121.1
50.0	94.9	116.0
Methylcholanthrene positive control		
2 µg/ml	7.4	923.9 <sup>d</sup>

<sup>a</sup>Relative growth = (relative suspension growth × relative cloning efficiency)/100.

<sup>b</sup>Mutant frequency = (total mutant colonies/total viable colonies) × 2 × 10<sup>-4</sup>. Decimal is moved to express the frequency in units of 10<sup>-6</sup>.

<sup>c</sup>Mutagenic as judged by exceeding the minimum criterion of 165.2 for this experiment (twice the control frequency).

<sup>d</sup>Mutagenic as judged by exceeding the minimum criterion of 228.6 for this experiment (twice the control frequency).

Table VII. Induction of chromosomal aberrations in CHO cells

Concentration	Aberrations per cell	Percent cells with aberrations <sup>a</sup>	Polyploid cells <sup>a</sup>	Endo-reduplicated cells <sup>a</sup>
20 h exposure without added metabolic activation				
AQ-DA ( $\mu\text{g/ml}$ )				
0.0	0.03	2.0	1.5	0.0
12.5	0.01	0.5	0.5	0.5
25.0	0.01	0.5	1.0	0.0
37.5	0.00	0.0	1.0	0.0
50.0	0.01	1.0	0.5	0.0
44 h exposure without added metabolic activation				
AQ-DA ( $\mu\text{g/ml}$ )				
0.0	0.01	0.5	1.0	0.0
12.5	0.00	0.0	2.5	0.0
25.0	0.01	1.0	6.5 <sup>a,b</sup>	0.0
37.5	0.00	0.0	4.5 <sup>a,b</sup>	0.0
50.0	0.03	2.5	6.0 <sup>a,b</sup>	0.0
20 h exposure without added metabolic activation				
Mitomycin C positive control				
0.1 $\mu\text{g/ml}$	0.30	28.0 <sup>a</sup>	1.5	0.0
20 h exposure with added metabolic activation				
AQ-DA ( $\mu\text{g/ml}$ )				
0.0	0.01	1.0	2.5	1.0
12.5	0.00	0.0	1.5	0.0
25.0	0.00	0.0	0.0	0.0
37.5	0.01	0.5	2.0	0.0
50.0	0.01	1.0	0.0	0.0
44 h exposure with added metabolic activation				
AQ-DA ( $\mu\text{g/ml}$ )				
0.0	0.00	0.0	2.0	0.0
12.5	0.00	0.0	1.0	0.0
25.0	0.01	1.0	1.0	0.0
37.5	0.00	0.0	1.0	0.0
50.0	0.00	0.0	1.5	0.0
20 h exposure with added metabolic activation				
Cyclophosphamide (CP) positive control				
5.0 $\mu\text{g/ml}$	0.46	32.0 <sup>a</sup>	2.0	0.0

<sup>a</sup>A test article was considered positive for inducing chromosomal aberrations if a significant increase was observed compared to controls at a level of  $P < 0.01$ .

<sup>b</sup>The weak increase in polyploidy observed in these cultures was judged a statistical anomaly because the values observed are within the historical negative control data of 0–9.5% and the solvent control data of 0–10% and there was no indication of activity under any of the other exposure conditions or trials.

either with or without metabolic activation in the Ames tester strains or in WP2uvrA (Tables IV and V).

#### L5178Y TK +/- mouse lymphoma forward mutation assay

No cytotoxicity was observed in any of the trials in the L5178Y mutation assays. Mutant frequencies of treated cultures varied randomly with dose toxicity and no increases above the minimum criteria for a positive response were induced (Table VI). AQ-DA was therefore evaluated as negative with and without metabolic activation at the TK locus in L5178Y mouse lymphoma cells under the conditions used in this study.

#### Chromosomal aberration induction in CHO cells

CHO cells were incubated with up to 50.0  $\mu\text{g/ml}$  of AQ-DA with harvest times of 20 and 44 h (Table VII). No significant increase in cells with chromosomal aberrations, polyploidy or endoreduplication was observed at the concentrations analyzed. AQ-DA was considered negative for inducing chromosomal aberrations in CHO cells with and without metabolic activation.

#### In vivo bone marrow mouse micronucleus assay

In the micronucleus assay, AQ-DA was suspended in corn oil and dosed by oral gavage at 1250, 2500 and 5000 mg/kg. No bone marrow toxicity was observed as a decrease in the

polychromatic erythrocyte: normochromatic erythrocyte (PCE:NCE) ratio. AQ-DA did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes under the conditions of this assay and is considered negative in the mouse bone marrow micronucleus test (Table VIII).

## Discussion

### Genetic toxicology

The lack of mutagenic or genotoxic activity in a variety of assays in numerous laboratories indicates that AQ is not a DNA-reactive genotoxic carcinogen (Brown and Brown, 1976; Anderson and Styles, 1978; Gibson *et al.*, 1978; Salamone *et al.*, 1979; Sakai *et al.*, 1985; Tikkanen *et al.*, 1983; National Cancer Institute, 1987; Kerckaert *et al.*, 1996; Durant *et al.*, 1996). Those assays are strengthened by the studies presented here in which we report that purified NTP AQ-OX, AQ-FC and AQ-DA are negative in the expanded Ames mutagenicity test, that AQ-DA is negative in the L5178Y mouse lymphoma forward mutation assay, that AQ-DA does not induce chromosomal aberrations, polyploidy or endoreduplication in CHO cells, and that it is negative in the *in vivo* mouse micronucleus assay (Tables III–VIII).

**Table VIII.** *In vivo* bone marrow micronucleus assay

Treatment	Dose (mg/kg)	Harvest time (h)	% Micronucleated PCEs Mean of 1000 per animal $\pm$ SE	
			Males	Females
			Corn oil	24
	48	0.02 $\pm$ 0.02	0.14 $\pm$ 0.05	
	72	0.16 $\pm$ 0.05	0.02 $\pm$ 0.02	
AQ-DA	1250	24	0.06 $\pm$ 0.04	0.10 $\pm$ 0.03
	1250	48	0.06 $\pm$ 0.04	0.12 $\pm$ 0.05
	1250	72	0.04 $\pm$ 0.02	0.08 $\pm$ 0.06
	2500	24	0.02 $\pm$ 0.02	0.16 $\pm$ 0.07
	2500	48	0.06 $\pm$ 0.02	0.02 $\pm$ 0.02
	2500	72	0.06 $\pm$ 0.02	0.02 $\pm$ 0.02
	5000	24	0.02 $\pm$ 0.02	0.04 $\pm$ 0.04
	5000	48	0.06 $\pm$ 0.02	0.06 $\pm$ 0.04
	5000	72	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Cyclo-phosphamide	80	24	4.94 $\pm$ 0.72 <sup>a</sup>	3.00 $\pm$ 1.04 <sup>a</sup>

<sup>a</sup>Significantly greater than the corresponding vehicle control at the  $P < 0.01$  level.

The report of direct-acting genotoxic activity in two Ames tests (Lieberman *et al.*, 1982; Zeiger *et al.*, 1988) was unexpected because the data were in conflict with so many other reports of negative mutagenic activity. In both of those reports the AQ was obtained from Aldrich Chemical Co. and the label purity was listed as only 97% (Lieberman *et al.*, 1982; Zeiger *et al.*, 1988). No analysis of the identity of the remaining 3% of non-AQ material was done. The problem of contamination with nitroanthracenes producing genotoxic activity in preparations of AQ-OX has been described (US EPA, 1977; ICI, 1978a, 1978b). In fact, the NTP AQ-OX was contaminated with 1200 p.p.m. 9-NA (Table I). The NTP AQ-OX was mutagenic in strains TA98, TA100 and TA1537, while the purified NTP AQ-OX was not. The structure of AQ does not suggest direct DNA reactivity, yet the NTP AQ-OX was mutagenic without added metabolic activation. Taken together these data indicate that AQ is not genotoxic, rather that the NTP sample of AQ-OX contains the mutagenic contaminant 9-NA.

#### Cytogenetics

The NTP report described a mouse peripheral blood micronuclei assay test from the 14 week range-finding study that preceded the cancer bioassay (NTP, 1999). The tentative conclusion was that the data showed that AQ-OX exhibited weak activity in that assay. However, the doses used were up to four times the maximum tolerated dose used in the bioassay, no response was seen in the female animals, and the response in the males was judged as positive only with a highly non-conservative trend test. In contrast, AQ-OX was negative in a bone marrow micronucleus assay (NTP, 1999). AQ-DA was also negative in a mouse bone marrow micronucleus assay in the studies reported here (Table VIII) and a chromosomal aberration assay in CHO cells (Table VII). Taken together, the weight of evidence of the data indicates that AQ does not induce cytogenetic damage.

#### Cancer studies

The observation of a mutagenic contaminant confounds any interpretation of the NTP bioassay with AQ. A previous bioassay with males and females in two strains of mice had been conducted with AQ. That study was not done following contemporary bioassay standards and needed to be repeated.

Nevertheless that bioassay did not show carcinogenic activity with AQ (Innes *et al.*, 1969). Looking at the data as a whole strongly indicates the possibility that the observed tumors in the NTP bioassay cancer were the result of a mutagenic contaminant.

Pathological evaluations of tissues from the NTP study suggest that there may have been some degree of cell death and regenerative cell proliferation in some target tissues. For example, in the rat kidneys from AQ-treated animals hyaline droplet accumulation, nephropathy, transitional epithelium hyperplasia and mineralization were observed. Centrilobular hypertrophy and focal necrosis were seen in the male B6C3F<sub>1</sub> mice livers, and centrilobular hypertrophy and focal fatty degeneration were seen in the female B6C3F<sub>1</sub> mice livers (NTP, 1999). In follow up studies in F-344 rats, AQ-induced cell proliferation was noted in the urinary bladder (National Institute of Environmental Health Sciences, 1999). If 9-NA or other mutagenic contaminants were present, even a small amount of regenerative cell proliferation would act synergistically to enhance the mutagenic and carcinogenic responses (Columbano *et al.*, 1981).

#### Contaminants as confounders of the cancer bioassay

The best way to evaluate the plausibility as to whether the contaminant in the NTP AQ-OX produced the tumor response in the NTP study would be to do a potency calculation based on the carcinogenic potency of the 9-NA contaminant. Unfortunately, no cancer data are available for 9-NA. One way to estimate potency is to extrapolate comparisons of genotoxic potency to potential carcinogenic potency. Although it is recognized that there are limitations to such comparisons, a reasonable estimate of plausibility can usually be made. Another way to address the plausibility question is to ask how potent a carcinogen the contaminant would have to be in order to produce the observed response.

#### Mutagenic potency comparisons

Both 9-NA and 2-nitroanthracene (2-NA) are mutagenic with the 2-NA isomer being substantially more potent (Fu *et al.*, 1986). 9-NA is present in the NTP AQ-OX at a level of 1200 p.p.m. Therefore, the amount of the contaminant present on each Ames test plate is easily calculated (shown in Table II). Since AQ is not mutagenic, all the mutagenic activity can be ascribed to the contaminants. Table II shows that mutagenic activity can be seen in strain TA98 without metabolic activation at a level of only 0.15  $\mu$ g 9-NA per plate. As a first approximation it is reasonable to assign this mutagenic activity to the primary contaminant 9-NA. Accordingly, on the basis of induced revertants per microgram in tester strain TA98, 9-NA with 173 revertants/ $\mu$ g was more potent than B[a]P with 137 revertants/ $\mu$ g and was as potent as the 2-nitrofluorene with 175 revertants/ $\mu$ g run concurrently as positive controls (Table II).

#### Cancer potency calculations

A critical question is whether it is plausible that a contaminant could be a significant contributor to the carcinogenic activity observed in the NTP bioassay. The *Handbook of Carcinogenic Potency and Genotoxicity Databases* defines a valuable parameter to rank cancer potency, the Tumor Dose 50 (TD<sub>50</sub>) (Gold and Zeiger, 1997). The TD<sub>50</sub> is the tumorigenic dose-rate for 50% of experimental animals, or the dose-rate that will halve the probability of remaining tumor free at the end of a standard lifespan. For a given target site, if there are no

Table IX. TD<sub>50</sub> values

Gender species tumor type	TD <sub>50</sub> assuming only AQ induced the tumors (mg/kg/day) <sup>a</sup>	TD <sub>50</sub> assuming only 9-NA induced the tumors (mg/kg/day) <sup>b</sup>
Male F344 rats	940	1.1
Renal tubule adenoma Male F344 rats	810	0.97
Bladder transitional epithelial papiloma Female F344 rats	310	0.37
Renal tubule transitional adenoma or carcinoma Male B6C3F <sub>1</sub> mice	380	0.45
Hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma Female B6C3F <sub>1</sub> mice Hepatocellular adenoma or hepatocellular carcinoma	390	0.47

<sup>a</sup>The TD<sub>50</sub> for key target sites in the NTP AQ cancer study were estimated from a least squares linear fit of the tumor data dose-response curve (Gold and Zeiger, 1997; NTP, 1999).

<sup>b</sup>The theoretical TD<sub>50</sub> for 9-NA was calculated knowing that the percentage of contaminating 9-NA was 0.12%, and assuming that the 9-NA was responsible for inducing the all the tumors.

tumors in control animals, then the TD<sub>50</sub> is that chronic dose rate in mg/kg body weight/day, which would induce tumors in half the test animals at the end of a standard lifespan study. This parameter is useful because it is analogous to the LD<sub>50</sub> and the units are understandable as mg/kg/day. The TD<sub>50</sub> for key target sites in the NTP AQ cancer study were estimated from a least squares linear fit of the dose-response tumor data and are presented in Table IX. Knowing that the percentage of contaminating 9-NA was 0.12%, and with the assumption that the 9-NA was responsible for inducing all the tumors, the resulting theoretical TD<sub>50</sub> for 9-NA was calculated and is also presented in Table IX. The TD<sub>50</sub> for AQ is in the range 310–940 for rats and is about 380 for mice. The AQ TD<sub>50</sub> potency in rats is in the range of butylated hydroxyanisole. If the 9-NA were responsible for all the tumorigenic activity, the TD<sub>50</sub> values would be in the range 0.37–1.1 for rats and would be about 0.45 for mice. Such values indicate that 9-NA would have to be in the potency range of B[a]P or 2-acetylaminofluorene in rats or dimethylnitrosamine or 3-nitro-3-hexene in mice (Gold and Zeiger, 1997). In fact, 9-NA has a greater mutagenic potency than B[a]P (Table II). These data indicate that it is plausible that the 9-NA contaminant was responsible for all of the tumor induction observed in the NTP study.

In fact, in the absence of reliable cancer data, the genetic toxicology profile indicates that AQ would not be a genotoxic carcinogen. Thus, no definitive conclusion can be drawn at this time as to whether AQ itself might exhibit carcinogenic activity.

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