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FYI-0400-001372

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April 5, 2000

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Subject: FYI Submission for 9,10-Anthracenedione, CAS # 84-65-1

Dear Sir or Madam:

The National Toxicology Program (NTP) has conducted studies of the compound 9,10-Anthracenedione, CAS # 84-65-1 (also known as Anthraquinone). These studies found clear evidence of carcinogenicity in rats and mice; the findings are reported in draft technical report 494. We have demonstrated that the sample of Anthraquinone employed by the NTP is contaminated by at least one mutagenic impurity which was responsible for part, if not all, of the carcinogenic activity observed in the NTP studies. We have written two similar papers detailing our findings; copies of these papers are enclosed with this letter.

TSCA submissions found at OTS 0521344 detail a positive mutagenic response obtained on one of six Anthraquinone samples submitted for bacterial mutagenicity testing; this single positive mutagenic response was found to be the result of contamination of the Anthraquinone sample with nitroanthracene resulting from the production process (oxidation of Anthracene to yield Anthraquinone). We submitted the NTP Anthraquinone sample for mutagenicity testing along with three other samples of Anthraquinone; only the NTP sample tested positive. Copies of the laboratory reports of the mutagenicity tests of these four samples are enclosed with this letter.

Analytical testing is being conducted to determine exactly which mutagenic impurities are present in the NTP Anthraquinone sample, and in what quantities. The presence of nitroanthracene in the NTP Anthraquinone sample has been confirmed. A further submission will be made when a full quantitative analysis of the NTP sample has been completed.

Dr Richard Irwin, the principal scientist for this technical report at NTP, and Dr Kenneth Olden, Director of NTP, have been informed of our findings.

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The enclosed documents discuss the very different production processes that can be employed to produce Anthraquinone. No Anthraquinone is produced in the U.S. at this time. The product of the oxidation of Anthracene represented less than 3% of the imports of Anthraquinone into the U.S. in 1999; the remainder was produced by the Friedel-Crafts process. Thus, the Anthraquinone in commerce in the U.S. would not be likely to be contaminated by the mutagenic impurities found in the NTP sample.

Sincerely,



Jerry A. Cook
Technical Director

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Impurities in 9,10 anthracenedione (Anthraquinone) sample administered by the National Toxicology Program in toxicity studies may have confounded study conclusions

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Abstract

Mutagenicity data are available for 18 samples of anthraquinone (including the 4 samples reported here); mutagenicity has been reported in only 3 samples. All of the previously published findings concerning bacterial mutagenicity of anthraquinone are tabulated here.

*Approximately 99% pure anthraquinone (AQ) powder administered by the National Toxicology Program in 2 year feeding studies in rats and mice (National Toxicology Program Technical Report Number 494, Toxicology and Carcinogenesis Studies of Anthraquinone) was not tested by the National Toxicology Program (NTP) for bacterial mutagenicity. AQ is stated in the report to be mutagenic in *Salmonella typhimurium* strains TA98 and TA100 with and without S9 metabolic activation enzymes based on the findings of Zieger et al. (1988) pertaining to a different sample of 97% purity AQ powder. A review of the other published reports concerning AQ mutagenicity shows that the Zieger et al. (1988) results are anomalous.*

A sample of the anthraquinone powder administered in the studies reported in Technical Report 494 was obtained from NTP and submitted to BioReliance, Inc. for bacterial mutagenicity testing, along with two samples of commercially available technical-grade AQ, and a purified reagent grade sample. This paper reports bacterial mutagenicity test results for these 4 samples. Only the NTP sample was mutagenic to bacterial tester strains TA98 or TA100, it was mutagenic in TA 98 with and without S9 metabolic activation, and was mutagenic in TA100 only without S9 metabolic activation.

EPA's TSCA submissions files for AQ contain bacterial mutagenicity information about 6 anthraquinone samples. All were found not to be mutagenic except for a sample of AQ powder which tested positive for bacterial mutagenicity in strains TA98 and TA1538 as a result of the presence of a mutagenic contaminant resulting from the manufacturing process (nitroanthracene). The impurity was destroyed by alkaline treatment; the sample was retested, and was found not to be mutagenic in either strains.

Our findings suggest that the AQ powder administered to rats and mice in the NTP toxicology and carcinogenicity studies reported in TR 494 is contaminated with a mutagenic impurity which has influenced the outcomes of the studies.

I. Introduction

Anthraquinone (AQ) is used as an intermediate for the production of dyes and pigments, and as a catalyst in the Kraft Process for the production of paper. AQ is insoluble in water and in dilute acid and alkaline solutions. AQ dissolves in concentrated sulfuric acid and this solution may be heated to 100°C without any reaction between the acid and the dissolved AQ; this characteristic has been employed to remove organic impurities from AQ (Allen's Commercial Organic Analysis, 1925).

AQ is produced in large quantities by at least three very different production methods in various parts of the world. The oxidation of anthracene to yield AQ is the oldest known production process and is now practiced primarily in Europe. 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 appears to be the most prevalent production method and is employed in China, India and other parts of the world. Production of AQ by the Diels-Alder reaction between 1,4-naphthoquinone and 1,3-butadiene is believed to be continuing in Japan. A fourth production method based on styrene was developed by BASF in the 1970's; it is reportedly being practiced in Eastern Europe.

There are visible differences in the appearance of the products of the different AQ production processes. The anthracene oxidation product is typically a golden yellow powder, whereas other production processes typically yield a tan or cream-colored powder. When sublimed, anthraquinone forms a pale yellow, crystalline material (Kirk-Othmer Encyclopedia of Chemical Technology, 1992).

While the great majority of the technical-grade AQ commercially available in the United States is produced by the Friedel-Crafts reaction, the available reagent grade material is believed to be almost exclusively the golden yellow product of direct oxidation of anthracene. The Kirk-Othmer Encyclopedia of Chemical Technology (1992) states concerning the anthracene oxidation process, "Unless the quality of the anthracene and the operating conditions are carefully controlled, the resulting anthraquinone may be contaminated."

Bacterial mutagenicity test results for AQ are contained in TSCA submissions (OTS 0521344) dating from the late 1970's and early 1980's. The submissions report the occurrence of an impurity, nitroanthracene, a known mutagen, in AQ produced by the direct oxidation of anthracene. AQ powder contaminated with 0.15% nitroanthracene was found to be mutagenic in *Salmonella typhimurium* mutagenicity tests; this same sample was subjected to an alkaline treatment known to destroy the nitroanthracene impurity, was retested, and was found not to be mutagenic. Five other samples of AQ powder produced by the direct oxidation of anthracene were tested at the same time and were found not to be mutagenic.

The National Toxicology Program (NTP) recently reported, in draft technical report number 494 (TR 494) that golden yellow AQ powder of about 99% purity obtained

from Zeneca Fine Chemicals produced clear evidence of carcinogenicity in mice and female rats in a 2-year feeding trial; this report underwent NTP's peer review on May 21, 1999 and was accepted as written, according to the information posted on the NTP web site. TR 494 states that AQ is mutagenic based upon the previous finding of mutagenicity in *Salmonella typhimurium* strains TA98 and TA100 from a study conducted by the National Toxicology Program on a sample of 97% purity AQ powder (Zeiger et al., 1988). This bacterial mutation assay was performed for NTP by Microbiological Associates, Inc. (now BioReliance, Inc.), Rockville, Maryland employing a preincubation assay protocol

Because our research had brought to light the contents of the EPA's TSCA file concerning the presence of a mutagenic impurity in a sample of AQ, the AQ sample administered to rats and mice in the toxicology and carcinogenicity studies reported in TR 494 was submitted for bacterial mutagenicity testing.

2. Materials and Methods

2.1. Chemicals

A sample of the AQ powder employed in the NTP 2-year toxicology and carcinogenesis studies was obtained from NTP, and was submitted for bacterial mutagenicity testing along with 2 samples of commercially available AQ produced by the Friedel-Crafts reaction in Chinese chemical facilities and a sample of reagent grade AQ which was further purified in our laboratory.

The golden yellow powder obtained from NTP, bearing a label stating "Anthraquinone, lot: 5893, CAS: 84-65-1, Store: RT", was stated in TR 494 to have been analyzed by NTP and found to be about 99% anthraquinone. A portion of the sample was submitted to BioReliance, Inc. for bacterial mutagenicity testing.

The two tan-colored powders, both technical-grade AQ powders produced by the Friedel-Crafts reaction at different facilities in China, were obtained from the Chinese companies, Zhou and Zhang Zhou, as being representative of commercially-available material. These samples were analyzed and found to be between 97% and 98% anthraquinone; they were submitted to BioReliance, Inc. at the same time as the sample obtained from NTP.

The golden yellow reagent grade AQ powder obtained from Eastman Fine Chemicals, Eastman Kodak Company was further purified by dissolution in concentrated sulfuric acid followed by reprecipitation upon dilution with hot water. A pale yellow powder was obtained upon reprecipitation, filtration, and drying; this was analyzed and found to be over 99% anthraquinone. This highly purified AQ powder was also submitted to BioReliance, Inc. for mutagenicity testing.

2.2. Bacterial Mutagenicity Assay

The four AQ powder samples described above were submitted to BioReliance, Inc., Rockville, MD, they were labeled only Quinone 1, Quinone 2, Quinone 3, and Quinone 4. BioReliance reported the physical descriptions of the samples in the final reports of their studies; these are in agreement with the descriptions of the samples given above. Quinone 1, the NTP sample, is described as a golden yellow powder; Quinones 2 and 3, the Chinese AQ samples, are described as tan powders; and Quinone 4, the purified reagent grade sample, is described as a light yellow powder.

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames et al. (1975). The test system was exposed to the test article via the preincubation methodology described by Yahagi et al. (1977). The test article was tested at eight dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate. One-half (0.5) milliliter of S9 or sham mix, 100 microliters of tester strain and 50 microliters of the dimethyl sulfoxide test vehicle control or containing the test article were added to 13 X 100 mm glass culture tubes pre-heated to $37\pm 2^\circ\text{C}$. After vortexing, these mixtures were incubated with shaking for 60 ± 2 minutes at $37\pm 2^\circ\text{C}$. Following the preincubation, 2.0 mL of selective top agar was added to each tube and the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50 microliter aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at $37\pm 2^\circ\text{C}$. Plates that were not counted immediately following the incubation period were stored at $2-8^\circ\text{C}$ until colony counting could be conducted. The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

For the test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of either one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA98 and TA100 were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

3.0 Results

The results of the mutagenicity assays are presented in Appendix I and summarized in Table I. With each of the four AQ powder samples tested, precipitate was observed at all doses of 250 micrograms per plate or greater. No appreciable toxicity was observed with any of the samples. Due to an unacceptable positive control value after the first evaluation, the response of tester strain TA100 in the absence of S9 activation was reevaluated for all four samples.

The sample obtained from NTP, and submitted to BioReliance as Quinone 1, caused positive responses with tester strains TA98 and TA100 in the absence of S9 activation and with tester strain TA98 in the presence of Arochlor-induced rat liver S9. None of the other samples caused positive responses with either of the tester strains.

Table I. A Compilation of all Available Bacterial Mutagenicity Test Results for Anthraquinone

Data Source	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Lieberman et al.	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG
Zeiger et al.	POS	POS	POS	POS						
Salamone et al.	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Brown and Brown	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Gibson et al.	NEG				NEG				NEG	
Anderson & Styles		NEG		NEG		NEG				NEG
Tikkanen et al.	NEG	NEG	NEG	NEG						
Sakai et al.	NEG	NEG	NEG	NEG						
Appendix A - NTP TR494 sample	POS	POS	POS	NEG						
Appendix A - Friedel-Crafts AQ from China	NEG	NEG	NEG	NEG						
Appendix A - Friedel-Crafts AQ from China	NEG	NEG	NEG	NEG						
Appendix A - Purified reagent grade AQ	NEG	NEG	NEG	NEG						
TSCA Submission OTS 0521344										
Sample	NEG	NEG	NEG	NEG	NEG	NEG			NEG	NEG
Sample B*	NEG	NEG	NEG	NEG	NEG	NEG			NEG	NEG
Sample C*	NEG	NEG	NEG	NEG	NEG	NEG			NEG	NEG
Sample D*	NEG	NEG	NEG	NEG	NEG	NEG			NEG	NEG
Sample E*	POS	POS	NEG	NEG	NEG	NEG			POS	POS
Sample F**	NEG	NEG	NEG	NEG	NEG	NEG			NEG	NEG
Sample E after alkaline treatment***	NEG	NEG	NEG	NEG	NEG	NEG			NEG	NEG

*Stated to have all been produced by the direct oxidation of anthracene but by three different processes.

**Stated to be reagent grade anthraquinone from Anachemia.

***Stated to have been found to be contaminated with 0.15% nitroanthracene as a result of the manufacturing process, then purified by alkaline treatment prior to this retest

4.0 Discussion

We believe that a positive *Salmonella typhimurium* mutagenicity test result for a sample of AQ powder demonstrates the presence of one or more mutagenic impurities; this conclusion is supported by the many negative test results for AQ powders found in our tests and reported elsewhere in the published literature. The only two previous studies reporting mutagenic activity for AQ powders (Lieberman et al., 1982; Zeiger et al., 1988) both obtained the powder tested from Aldrich Chemical Company. Zeiger et al. (1988) is the only previous report of mutagenicity in strain TA100.

Bacterial mutagenicity test results for 18 different samples of AQ are presented in Table I. Appendix A contains specific details of the test results on the four samples submitted to BioReliance, Inc. for bacterial mutagenicity testing.

EPA's TSCA submissions docket contains information which is highly relevant to the apparent variability in bacterial mutagenicity test results for AQ. As shown in Table I, a sample of AQ, stated in the submission to have been produced by the oxidation of anthracene, was found to be mutagenic, was then found to contain a mutagenic impurity (stated to be 0.15% nitroanthracene), and, upon retesting, was found not to be mutagenic after the impurity was removed (OFS 0521344). This sample is not considered to have been mutagenic because the cause of the positive test response was identified as being a substance other than anthraquinone.

The draft of TR 494 makes no mention of the bacterial mutagenicity information concerning AQ contained in the TSCA submissions. The report discusses the variation in the published findings concerning the mutagenicity of various AQ samples as follows: "Early mutagenicity studies of anthraquinone in *Salmonella typhimurium*, most using the plate incorporation assay protocol, reported negative results (Brown and Brown, 1976; Anderson and Styles, 1978; Gibson et al., 1978; Salamone et al., 1979; Tikkanen et al., 1983; Sakai et al., 1985). Later studies showed clear mutagenic activity for anthraquinone in TA100 and the frameshift strains TA98, TA1537, and TA1538; in the presence and absence of S9 activation enzymes (Lieberman et al., 1982; Zeiger et al., 1988). None of the bacterial mutagenicity assays that reported negative results included the purity of the anthraquinone samples used for testing. The Zeiger et al. (1988) preincubation assay that produced positive results tested an anthraquinone sample that was 97% pure. Sample purity, along with dose selection and other protocol variations, may have been critical to the outcome of these mutagenicity assays."

The mutagenicity of the sample of AQ powder obtained from NTP demonstrates, in our opinion, that the findings reported in TR 494 are most probably confounded by the presence of a mutagenic impurity contaminating the AQ powder fed to rats and mice. NTP did not determine the identities of the substances composing about 1% of the powder tested; this should be done in an effort to learn which mutagen is present in the powder.

Unfortunately, some other published findings concerning AQ are called into question by our findings concerning the NTP sample. Dose-related increases in

micronuclei were reported in cultured Syrian hamster embryo cells treated with 3.13 to 25 micrograms/ml of this same TR494 anthraquinone sample obtained from NTP (Gibson et al., 1997). In contrast, a different AQ powder was tested for induction of forward mutations in cultured human BT lymphoblastoid cells, a metabolically competent cell line for polycyclic aromatic compounds (Durant et al., 1996), and no mutagenic activity was detected.

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Zeiger, E; Haseman, JK; Shelby, MD; Margolin, BH, and Tennant, RW (1988) Evaluation of four in vitro genetic toxicity tests for predicting rodent carcinogenicity: Confirmation of earlier results with 41 additional chemicals. *Environ. Mol. Mutagen.* 16 (Suppl. 18), 1-14.

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**Mutagenic Impurities in the Test Material May Have Been Responsible for the
Carcinogenic Activity Observed in the National Toxicology Program Bioassay with
Anthraquinone**

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Submitted to *Mutation Research* for publication, February, 2000

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Abstract

Anthraquinone (AQ) (9,10-anthracenedione) has been reported to be negative in a variety of genotoxicity tests including numerous Ames Salmonella mutagenicity assays. Nevertheless, in a long-term study conducted by the National Toxicology Program (NTP) AQ induced a weak to modest dose related increase in tumors in the kidney and bladder of male and female F344/N rats and in the livers of female B6C3F₁ mice. Commercial AQ is produced by at least three different production methods worldwide, with the final product varying in color and purity. In the studies reported here, four samples of AQ were subjected to the Ames bacterial mutagenicity assay in the same laboratory at the same time. These included a sample of the AQ used in the NTP bioassay, two samples produced in factories using the Friedel-Crafts reaction, and purified reagent grade AQ. The only positive responses observed were with the NTP bioassay material, this sample exhibited mutagenic activity in strains TA98 and TA100 without metabolic activation. Addition of an S-9 metabolic activation system decreased or eliminated the mutagenic activity. The chemical structure of AQ does not suggest that the parent compound would be DNA reactive. An earlier 18 month bioassay conducted with AQ administered to male and female B6C3F₁ and (C57BL/6 X AKR)F₁ mice reported no induction of cancer. Therefore, it appears that a mutagenic contaminant is present in the NTP bioassay sample that is either directly mutagenic or can be activated by bacterial metabolism. An earlier study also reported the observation of a mutagenic contaminant in a sample of AQ. Therefore, it is likely that part, if not all, of the carcinogenic activity observed in the NTP AQ bioassay was the result of

mutagenic contaminants, rather than the AQ. The Ames test can be a valuable tool to determine the presence of mutagenic contaminants and to avoid AQ production processes that would produce potential environmental carcinogens.

Keywords: anthraquinone, mutagenic impurities, Ames salmonella mutagenicity assay

1. Introduction

Anthraquinone (AQ) (9,10-anthracenedione) is used as an intermediate for the production of dyes and pigments, and as a catalyst in the Kraft Process for the production of paper. AQ is produced in large quantities by at least three different production methods in various parts of the world. The oxidation of anthracene to yield AQ is the oldest known production process and is now practiced primarily in Europe. 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 appears to be the most prevalent production method and is employed in China, India and other parts of the world. Production of AQ by the Diels-Adler reaction between 1,4-naphthoquinone and 1,3-butadiene is believed to be continuing in Japan. A fourth production method based on styrene was developed by BASF Corp. in the 1970's and is reportedly practiced to some extent in Eastern Europe.

There are visible differences in the appearance of the products of the different AQ production processes. The anthracene oxidation product is typically a golden yellow powder, whereas other production processes typically yield a tan or cream-colored powder. When sublimed, anthraquinone forms a pale yellow, crystalline material [1]. While the great majority of the technical-grade AQ commercially available in the United States is produced by the Friedel-Crafts reaction, the available reagent grade material is believed to be almost exclusively the golden yellow product of direct oxidation of anthracene. In the oxidation of anthracene to AQ, the resulting anthraquinone product may be contaminated unless the quality of the anthracene and the operating conditions are carefully controlled [1].

AQ tested negative in the Syrian hamster embryo (SHE) cell transformation assay [9]. AQ is also not mutagenic in a line of human B-lymphoblastoid cells that constitutively express cytochrome P4501A1 [10]. An 18-month bioassay conducted with AQ administered to male and female B6C3F1 and (C57BL/6 X AKR)/F1 mice reported no induction of cancer [11]. Thus, it was somewhat unexpected when the National Toxicology Program (NTP) reported in Technical Report 494 that AQ induced a weak to modest dose related increase in tumors in the kidney and bladder of male and female F344/N rats and in the livers of female B6C3F₁ mice [12].

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 [2-8]. In contrast to the numerous papers documenting a lack of AQ mutagenic activity as 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 S-9 metabolic activation system reduced or eliminated the response [13, 14]. The chemical structure of AQ does not suggest that the parent compound would be a DNA reactive mutagen. Therefore, it seems likely that a mutagenic contaminant was present in the positive Ames test samples that was either directly mutagenic or could be activated by bacterial metabolism.

Further, bacterial mutagenicity test results for AQ are contained in TSCA submissions dating from the late 1970's [15]. In those studies, six samples of AQ were subjected to the Ames salmonella mutagenicity assay. Only one was positive; it was positive in TA 98 and TA1538 both with and without metabolic activation. It was determined that the mutagenic activity came from contamination of the sample with 0.15% nitroanthracene as result of the production process employed. When that sample was purified and retested, it showed no mutagenic activity.

It is clear that samples produced by different production methods can vary widely in the types and amounts of impurities associated with the AQ product. NTP did not perform bacterial mutagenicity testing on the AQ employed in its long-term study; a different sample of AQ had previously been tested by NTP [14]. The purpose of the present study was to determine the mutagenic activity of the actual material used in the NTP bioassay [12] and to contrast that with other commercially-available AQ products. A sample of the archived AQ sample tested in the NTP carcinogenicity studies was generously provided by the National Toxicology Program. The NTP AQ was contrasted in the Ames salmonella mutagenicity assay with samples produced in two currently

operating facilities using the Friedel-Crafts reaction, as well as purified reagent grade AQ. Assays were run under identical conditions in the same laboratory, at the same time.

2. Materials and Methods

2.1. Chemicals

A sample of the AQ powder employed in the NTP 2-year toxicology and carcinogenesis studies [12] was generously provided by Patricia Athey, NTP Chemical Management Leader, Battelle, Columbus OH. The golden yellow powder obtained from the NTP was labeled "Anthraquinone, Battelle Task Identifier: 4-064-SHIP-170, lot: 5893, CAS: 84-65-1, Store: RT". The technical report stated that this sample had been analyzed by NTP and was found to be about 99% anthraquinone [12]. This sample was designated NTP AQ.

Two samples of commercially available technical grade AQ produced by the Friedel-Crafts reaction were obtained from two different Chinese companies which supply technical grade AQ to Chemical Products Technologies, LLC in Cartersville, Georgia. These samples were designated as technical 1 AQ and technical 2 AQ. Both were tan-colored powders representative of commercially available material.

Golden yellow reagent grade AQ powder obtained from Eastman Fine Chemicals, Eastman Kodak Company was further purified by dissolution in concentrated sulfuric acid followed by reprecipitation upon dilution with hot water. A pale yellow powder was obtained upon reprecipitation, filtration and drying. This highly purified sample was designated pure AQ.

2.2. Bacterial Mutagenicity Assay

The four samples NTP AQ, technical 1AQ, technical 2 AQ, and pure AQ were coded and submitted to BioReliance Laboratories in Rockville, MD for evaluation in the Ames Salmonella mutagenicity assay according to their standard protocols. The tester strains used were the Salmonella typhimurium histidine auxotrophs TA98 and TA100 as described by Ames et al. [16]. The test system was exposed to the test article via the preincubation methodology described by Yahagi et al. [17]. The test article was tested at eight dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate.

Briefly, one-half (0.5) milliliter of S9 or sham mix, 100 microliters of tester strain and 50 microliters of the dimethyl sulfoxide test vehicle control or containing the test article were added to 13 X 100 mm glass culture tubes pre-heated to $37 \pm 2^\circ\text{C}$. After vortexing, these mixtures were incubated with shaking for 60 ± 2 minutes at $37 \pm 2^\circ\text{C}$. Following the preincubation, 2.0 mL of selective top agar was added to each tube and the mixture

was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50 microliter aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at $37 \pm 2^\circ\text{C}$. Plates that were not counted immediately following the incubation period were stored at $2-8^\circ\text{C}$ until colony counting could be conducted. The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

For the test article to be evaluated as positive, it must cause a dose-related increase in the mean revertants per plate of either one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA98 and TA100 were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than two times the mean vehicle control value.

3. Results

With each of the four AQ powder samples tested, precipitate was observed at all concentrations of 250 micrograms per plate or greater. The precipitate did not interfere

with scoring the plates. No appreciable toxicity was observed with any of the samples. The NTP AQ sample was mutagenic in a dose dependent manner in strains TA98 and TA100 (Table 1). Mutagenic activity was reduced or eliminated by addition of an S9 rat liver microsome metabolic activation system. No mutagenic activity was observed with the technical 1 AQ, technical 2 AQ, or pure AQ samples (data not shown).

4. Discussion

The lack of mutagenic or genotoxic activity in a variety of assays in numerous laboratories suggested that AQ would not be a DNA reactive genotoxic carcinogen [2-9]. When carcinogenic activity was observed in the NTP bioassay, an initial conclusion might be that AQ was acting via a nongenotoxic mode of action [18]. The observation of direct acting genotoxic activity in two studies [13, 14] was unexpected because the data were in conflict with so many other reports of negative mutagenic activity and because the chemical structure of AQ does not suggest direct DNA reactivity. AQ can be produced from different starting materials by different processes leading to products containing different impurities; this fact raised the possibility that mutagenic contaminants could be serious confounders in the biological activity of different preparations of AQ. In fact, TSCA submissions for AQ clearly demonstrate that a mutagenic impurity could be present in an AQ product and that it could affect the results of the bacterial mutagenicity assay [15]

A previous bioassay with males and females in two strains of mice had been conducted with AQ. This study was not done following contemporary bioassay standards and needed to be repeated. Nevertheless that bioassay did not show carcinogenic activity with AQ [11]. Looking at the data as a whole strongly suggested the possibility that the NTP bioassay cancer results might also be the result of mutagenic contaminants.

Indeed, results in this study show that only the NTP AQ sample, and not the technical 1 AQ, the technical 2 AQ, or the pure AQ samples, is mutagenic in tester strains TA98 and TA100. Mutagenic activity was reduced or eliminated with the addition of an S9 activation system (Table 1). This indicates the presence of a mutagenic impurity in the NTP AQ sample that is either directly DNA reactive or is activated by bacterial metabolism. The NTP AQ sample was also positive in inducing micronuclei in SHE cells [9]. These data suggest that part, if not all, of the carcinogenic activity of AQ reported in the NTP bioassay may have been induced by the direct-acting genotoxicant in the sample. Genotoxic carcinogens tend to induce cancer in multiple target organs, in both sexes, and across species [19]. This was the pattern produced by NTP AQ and is consistent with a direct acting mutagen. It is not the pattern seen with nongenotoxic carcinogens, where tumors tend to be induced only in tissues where there are preceding toxic events [18, 19]. The fact that mutagenic activity of the NTP AQ sample was readily measured, indicates that the impurity must be a fairly potent mutagen.

The definitive experiment would be to test different samples of technical grade and pure AQ for carcinogenic activity. Unfortunately, this is cost and time prohibitive.

One practical course of action is to utilize the Ames test as a tool to avoid AQ production processes that would produce potential environmental carcinogens.

Acknowledgments

We acknowledge the generous gift from C. W. Jameson and Larry Hart of the National Toxicology Program and Patricia Athey from Battelle of a sample of the AQ from their archives that was used in the NTP Cancer bioassay. Mutagenicity studies were conducted by Valentine O. Wagner, III and Sean M. Caruthers of BioReliance Laboratory in Rockville, MD. We especially thank Byron Butterworth for valued discussions and editing of the manuscript.

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Table 1. Activity of the NTP AQ sample in the Salmonella Mutagenicity Assay

Without liver microsomes (S9)		
Micrograms per plate	Average revertants per plate +/- SD	
	TA98	TA100
0	19 +/- 6	187 +/- 5
10	16 +/- 3	181 +/- 23
25	20 +/- 0	201 +/- 37
50	31 +/- 1	197 +/- 9
100	27 +/- 1	193 +/- 29
250	61 +/- 1	193 +/- 25
500	108 +/- 6	224 +/- 26
1000	127 +/- 4	267 +/- 18
2500	225 +/- 1	409 +/- 11
pos control	626 +/- 37	621 +/- 5

With liver microsomes (S9)		
Micrograms per plate	Average revertants per plate +/- SD	
	TA98	TA100
0	18 +/- 7	143 +/- 5
10	13 +/- 5	148 +/- 2
25	19 +/- 2	139 +/- 19
50	29 +/- 3	139 +/- 3
100	20 +/- 2	118 +/- 16
250	25 +/- 8	115 +/- 21
500	44 +/- 1	115 +/- 10
1000	52 +/- 8	123 +/- 8
2500	115 +/- 21	144 +/- 22
pos control	840 +/- 95	455 +/- 28

FINAL REPORT

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Study Title

Salmonella Preincubation Mutagenicity Assay

Test Article

Quinone 1

*N.T.P. Sample
Employed in
studies reported
in draft TR494*

Sponsor Project Number

5893

Authors

Valentine O. Wagner III, M.S.
Sean M. Caruthers, B.S.

Study Completion Date

December 22, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

VA221J801004 BFI

Sponsor

Chemical Products Corporation
102 Old Mill Road SE
Lawrenceville, GA 30046

Salmonella Preincubation Mutagenicity Assay

FINAL REPORT

Sponsor: Chemical Products Corporation
102 Old Mill Road SE
Cartersville, GA 30120

Authorized Representative: Mr. Jerry Cook

Performing Laboratory: BioReliance
9630 Medical Center Drive
Rockville, Maryland 20850

Test Article I.D.: Quinone 1

Sponsor Project No.: 5893

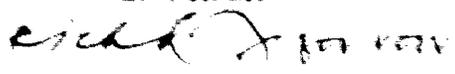
BioReliance Study No.: AA221J.501004.BTL

Test Article Description: golden yellow powder

Storage Conditions: room temperature; protected from exposure to light and moisture

Test Article Receipt: 05 October 99

Study Initiation: 20 October 99

Study Director:  22 Dec 1999

Valentine O. Wagner, III, M.S. Date

EXPERIMENTAL DESIGN AND METHODOLOGY

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975). Tester strain TA98 is reverted from auxotrophy to prototrophy by frameshift mutagens. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Experimental Design

The test system was exposed to the test article via the preincubation methodology described by Yahagi *et al.* (1977). The test article was tested at a minimum of eight dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate.

Plating and Scoring Procedures

In the preincubation method, one-half (0.5) milliliter of S9 or sham mix, 100 μ L of tester strain and 50 μ L of vehicle or test article were added to 13 X 100 mm glass culture tubes pre-heated to $37 \pm 2^\circ\text{C}$. After vortexing, these mixtures were incubated with shaking for 60 ± 2 minutes at $37 \pm 2^\circ\text{C}$. Following the preincubation, 2.0 mL of selective top agar was added to each tube and the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50 μ L aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at $37 \pm 2^\circ\text{C}$. Plates that were not counted immediately following the incubation period were stored at -8°C until colony counting could be conducted.

The condition of the bacterial background flora was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Evaluation of Results

For the test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate for either one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA98 and TA100 were judged positive if the increase in mean revertants at the peak of the dose response is greater or greater than two times the mean vehicle control value.

RESULTS AND DISCUSSION

Solubility

Dimethyl sulfoxide was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells. Concentrations from 0.20 to 50 mg/mL were delivered to the test system as workable suspensions.

Mutagenicity Assay

The results of the mutagenicity assay are presented in Tables 1 through 4 and summarized in Table 5. These data were generated in Experiments B1 and B2. Precipitate was observed at ≥ 250 μg per plate. No appreciable toxicity was observed.

In Experiment B1, positive responses were observed with tester strain TA98 in the presence (6.4-fold, maximum increase) and absence (11.8-fold, maximum increase) of S9 activation. No positive response was observed with tester strain TA100 in the presence of S9 activation. Due to an unacceptable positive control value, tester strain TA100 in the absence of S9 activation was not evaluated but was retested in Experiment B2.

In Experiment B2, a positive response was observed with tester strain TA100 in the absence (2.2-fold, maximum increase) of S9 activation.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the *Salmonella* Preincubation Mutagenicity Assay indicate that under the conditions of this study, **Quinone 1** did cause positive responses with tester strains TA98 and TA100 in the absence of S9 activation and with tester strain TA98 in the presence of Aroclor-induced rat liver S9.

Salmonella Mutagenicity Assay

Table 1

Test Article Id	Quinone		Experiment No.	E1	
Study Number	AA2111 90-004 ETL		Cells Seeded	2.6 X 10 ⁸	
Strain	TA98		Date Plated	10/26/99	
Liver Microsomes	None		Counted by	hand	
Vehicle	dimethyl sulfoxide (DMSO)				
Plating Aliquot	50 µL				
Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	23	1	19	6
	02	14	1		
10	01	18	1	16	7
	02	15	1		
25	01	21	1	20	6
	02	19	1		
50	01	30	1	29	11
	02	22	1		
100	01	28	1	28	11
	02	28	1		
250	01	3	INF	1	-
	02	0	INF		
500	01	11	INF	6.5	7
	02	12	INF		
1000	01	11	INF	11.5	7
	02	13	INF		
Average (mean) ± standard deviation			µg per plate		
Vehicle			19 ± 6		
10			16 ± 7		
25			20 ± 6		
50			29 ± 11		
100			28 ± 11		
250			1 ± -		
500			6.5 ± 7		
1000			11.5 ± 7		

^a Background Code: 1 = 1 revertant per plate; INF = insufficient for counting; - = no revertants observed.

Salmonella Mutagenicity Assay

Table 2

Test Article Id : Quinone 1
 Study Number : AA22JJ.501004.BTL Experiment No : 31
 Strain : TA98 Cells Seeded : 2.6×10^8
 Liver Microsomes : Rat liver S9 Date Plated : 10/26/99
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 50 μ L Counted by : hand

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code*	Average Revertants	Standard Deviation
Vehicle	01	13	1	18	7
	02	23	1		
16	01	9	1	13	5
	02	16	1		
25	01	20	1	19	2
	02	17	1		
50	01	27	1	24	3
	02	31	1		
160	01	18	1	20	2
	02	21	1		
250	01	31	1NP	25	5
	02	19	1NP		
500	01	33	1NP	33	1
	02	41	1NP		
1000	01	33	1NP	33	5
	02	47	1NP		
2500	01	100	1NP	100	1
	02	100	1NP		
Positive control 2-aminanthracene 1.0 μ g per plate					
	01	107	1		
	02	113	1		

*Background level of revertants per plate
 *Notes: 1. Susceptible to phage
 2. F⁻ cells used
 3. F⁻ cells used
 4. F⁻ cells used

Salmonella Mutagenicity Assay

Table 1

Test Article Id: Quinone 1
 Study Number: AA223J 001004 BFL
 Experiment No: B2
 Strain: TA100
 Cells Seeded: 0.8×10^8
 Liver Microsomes: None
 Date Plated: 11/23/99
 Vehicle: dimethyl sulfoxide (DMSO)
 Plating Aliquot: 50 μ L
 Counted by: machine

Concentration ug per plate	Plate Number	Revertants per plate	Background Code*	Average Revertants	Standard Deviation
Vehicle	01	28	1	28.0	5
	02	8	1		
1	01	177		181	21
	02	185			
2.5	01	177		177	21
	02	177			
50	01	177		177	21
	02	177			
100	01	177		177	21
	02	177			
500	01	INF	INF	INF	INF
	02	INF	INF		
1000	01	INF	INF	INF	INF
	02	INF	INF		
DMSO	01	INF	INF	INF	INF
	02	INF	INF		

*Background Code: 1 = background count is less than 10 revertants per plate; 2 = background count is between 10 and 20 revertants per plate; 3 = background count is between 20 and 30 revertants per plate; 4 = background count is between 30 and 40 revertants per plate; 5 = background count is between 40 and 50 revertants per plate; 6 = background count is between 50 and 60 revertants per plate; 7 = background count is between 60 and 70 revertants per plate; 8 = background count is between 70 and 80 revertants per plate; 9 = background count is between 80 and 90 revertants per plate; 10 = background count is between 90 and 100 revertants per plate; INF = background count is infinite (no revertants counted).

Salmonella Mutagenicity Assay

Table 4

Test Article Id : Quinone 1
 Study Number : AA22JJ.501004.BTL Experiment No : 81
 Strain : TA100 Cells Seeded : 0.9×10^8
 Liver Microsomes : Rat liver S9 Date Plated : 10/26/99
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 50 μ L Counted by : machine

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	146	1	146	5
	02	139	1		
10	01	149	1	149	5
	02	146	1		
25	01	125	1	125	13
	02	152	1		
50	01	137	1	137	5
	02	141	1		
100	01	129	1	129	5
	02	106	1		
250	01	122	INF	122	5
	02	100	INF		
500	01	122	INF	122	13
	02	109	INF		
1000	01	127	INF	127	5
	02	123	INF		
2500	01	150	INF	150	5
	02	118	INF		

^aPositive Control: Thymineless medium (Tm) 100% (100%)

01 = 100%

02 = 100%

^bBackground: Thymineless medium (Tm) 100% (100%)

01 = 100%

02 = 100%

INF = Not Informed

Salmonella Mutagenicity Assay
Summary of Results

Table 1

Test Article Id	Quinone 1	Experiment Nos
Study Number	AA22JU 50100, BTL	81/82
Average Revertants Per Plate ± Standard Deviation		
Liver Microsomes	None	
Dose (µg)	TA98	TA100 ^a
0.0	10 ± 6	157 ± 20
10	16 ± 3	151 ± 23
20	20 ± 7	201 ± 37
50	27 ± 10	197 ± 27
100	27 ± 10	197 ± 26
250	31 ± 7	199 ± 18
500	48 ± 11	204 ± 16
1000	27 ± 7	197 ± 18
2500	228 ± 11	409 ± 11
POS	62 ± 7	121 ± 5
Liver Microsomes: Rat Liver S ₉		
Dose (µg)	TA98	TA100 ^a
0.0	18 ± 6	117 ± 8
10	17 ± 8	124 ± 17
20	17 ± 7	126 ± 20
50	21 ± 6	122 ± 17
100	20 ± 7	118 ± 17
250	27 ± 7	121 ± 21
500	27 ± 8	115 ± 17
1000	31 ± 8	116 ± 18
2500	118 ± 11	240 ± 22
POS	32 ± 6	131 ± 18

^a TA100 is a derivative of TA98. The number of revertants per plate for TA100 is approximately 10% higher than for TA98. The number of revertants per plate for TA100 is approximately 10% higher than for TA98.

FINAL REPORT

Study Title

Salmonella Preincubation Mutagenicity Assay

Test Article

Quinone 2

*Friedel - Crafts
Anthraquinone*

Sponsor Project Number

5893

Authors

Valentine O. Wagner, III, M.S.
Sean M. Caruthers, B.S.

Study Completion Date

December 22, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

AA22JK.501004 BT1

Sponsor

Chemical Products Corporation
102 Old Mill Road SE
Cartersville, GA 30120

Salmonella Preincubation Mutagenicity Assay

FINAL REPORT

Sponsor: **Chemical Products Corporation**
102 Old Mill Road SE
Cartersville, GA 30120

Authorized Representative: **Mr. Jerry Cook**

Performing Laboratory: **BioReliance**
9630 Medical Center Drive
Rockville, Maryland 20850

Test Article ID: **Quinone 2**

Sponsor Project No: **5893**

BioReliance Study No: **AA22JK.501004.BT1**

Test Article Description: **tan powder**

Storage Conditions: **room temperature; protected from exposure to light and moisture**

Test Article Receipt: **05 October 99**

Study Initiation: **20 October 99**

Study Director: *Richard J. Wagner III* **22 Dec 1999**

 Valentine O. Wagner, III, M.S. Date

EXPERIMENTAL DESIGN AND METHODOLOGY

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975). Tester strain TA98 is reverted from auxotrophy to prototrophy by frameshift mutagens. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Experimental Design

The test system was exposed to the test article via the preincubation methodology described by Yahagi *et al.* (1977). The test article was tested at a minimum of eight dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate.

Plating and Scoring Procedures

In the preincubation method, one-half (0.5) milliliter of S9 or sham mix, 100 μ L of tester strain and 50 μ L of vehicle or test article were added to 13 X 100 mm glass culture tubes pre-heated to $37 \pm 2^\circ\text{C}$. After vortexing, these mixtures were incubated with shaking for 60 ± 2 minutes at $37 \pm 2^\circ\text{C}$. Following the preincubation, 20 mL of selective top agar was added to each tube and the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50 μ L aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at $37 \pm 2^\circ\text{C}$. Plates that were not counted immediately following the incubation period were stored at $2-8^\circ\text{C}$ until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Evaluation of Results

For the test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate for either the tester strains TA98 or TA100 with increasing concentrations of test article. Data sets for strains TA98 and TA100 were judged positive if the increase in mean revertants of the test article dose response is equal to or greater than two times the mean vehicle control response.

Salmonella Mutagenicity Assay

Table 1

Test Article Id : Quinone 2
 Study Number : AA22JK.501004.BTL Experiment No. : B1
 Strain : TA98 Cells Seeded : 2.6×10^8
 Liver Microsomes : None Date Plated : 10/26/99
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 50 μ L Counted by : hand

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code*	Average Revertants	Standard Deviation
Vehicle	01	23	1	10	5
	02	14	1		
10	01	11	1	14	7
	02	17	1		
25	01	1	1	5	2
	02	7	1		
50	01	12	1	10	2
	02	9	1		
100	01	18	1	12	5
	02	7	1		
250	01	11	1NP	11	5
	02	11	1NP		
500	01	6	1NP	11	5
	02	15	1NP		
1000	01	17	1NP	10	5
	02	21	1NP		
2500	01	12	1NP	12	5
	02	10	1NP		

Positive Control: 1000 μ g plate⁻¹ of TA98 with 100 μ g plate⁻¹ of S9

Background: 1000 μ g plate⁻¹ of TA98 with 100 μ g plate⁻¹ of S9
 1000 μ g plate⁻¹ of TA98 with 100 μ g plate⁻¹ of S9
 1000 μ g plate⁻¹ of TA98 with 100 μ g plate⁻¹ of S9
 Positive control: 1000 μ g plate⁻¹ of TA98 with 100 μ g plate⁻¹ of S9

Salmonella Mutagenicity Assay

Table 1

Test Article Id	Quinone	Experiment No	SI
Study Number	AAJLS-1144-5TL	Cells Seeded	2.6×10^5
Strain	FA38	Date Plated	10/26/99
Liver Microsomes	Rat Liver S9		
Vehicle	Dimethyl Sulfoxide (DMSO)		
Plating Aliquot	20 µL	Counted by	Hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
vehicle	01	17	L		
	02	14	L	15	1
10	1	17	L		
	2	17	L		
20	1	14	L		
	2	14	L		
30	1	14	L	14	1
	2	12	L		
100	1	14	L	17	3
	2	14	L		
150	1	14	DNF		
	2	14	DNF		
200	1	14	DNF		
	2	14	DNF		
250	1	14	DNF		
	2	14	DNF		
Positive Control	1	14	DNF		
	2	14	DNF	14	1
Blank	1	14	DNF		
	2	14	DNF		

Salmonella Mutagenicity Assay

Table 3

Test Article Id : Quinone 2
 Study Number : AA22JK.501004 BTL Experiment No. 82
 Strain : TA100 Cells Seeded 0.8 x 10⁸
 Liver Microsomes : None Date Plated 11-23-99
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 50 µL Counted by machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	190	1	190	5
	02	183	1		
10	01	177	1	177	10
	02	179	1		
25	01	175	1	175	10
	02	162	1		
50	01	212	1	212	14
	02	185	1		
100	01	158	1	158	10
	02	203	1		
250	01	162	1NF	162	10
	02	164	1NF		
500	01	158	1NF	158	10
	02	167	1NF		
1000	01	161	1NF	161	10
	02	160	1NF		
2500	01	167	1NF	167	10
	02	164	1NF		

POSTIVE CONTROL SYSTEM (10 µg/ml) (10⁸ CFU)

1 177

2 179

.....
 *ACKNOWLEDGEMENTS: The authors wish to thank the following individuals for their assistance in the performance of this study: [illegible]
 [illegible]
 [illegible]
 [illegible]

**Salmonella Mutagenicity Assay
Summary of Results**

Table 5

Test Article Id : Quinone 2
Study Number : AA22JK.501004.BTL **Experiment Nos:** B1/B2

Average Revertants Per Plate \pm Standard Deviation
Liver Microsomes: None

Dose (μ g)	TA98		TA100 ^a	
0.0	19 \pm 6	187 \pm 5		
10	14 \pm 4	178 \pm 1		
25	6 \pm 2	164 \pm 30		
50	11 \pm 2	199 \pm 19		
100	13 \pm 8	181 \pm 32		
250	11 \pm 0	163 \pm 1		
500	11 \pm 6	205 \pm 4		
1000	19 \pm 3	206 \pm 8		
2500	17 \pm 3	206 \pm 19		
Pos	626 \pm 47	621 \pm 5		

Liver Microsomes: Rat Liver S9

Dose (μ g)	TA98		TA100	
0.0	18 \pm 7	143 \pm 5		
10	20 \pm 4	130 \pm 21		
25	13 \pm 1	103 \pm 11		
50	24 \pm 2	130 \pm 6		
100	27 \pm 4	150 \pm 2		
250	23 \pm 6	145 \pm 18		
500	26 \pm 1	138 \pm 24		
1000	24 \pm 8	117 \pm 4		
2500	27 \pm 2	115 \pm 24		
Pos	360 \pm 35	355 \pm 28		

^a = vehicle control, 100 μ g/ml

Pos = Positive Control (see experiment 55 for details of Method and Materials)

* = Data from Experiment 57

FINAL REPORT

Study Title

Salmonella Preincubation Mutagenicity Assay

Test Article

Quinone 3

*Friedel-Crafts
Anthraquinone*

Sponsor Project Number

5893

Authors

Valentine O. Wagner, III, M.S.
Sean M. Caruthers, B.S.

Study Completion Date

December 22, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

XX22H-501004-B11

Sponsor

Chemical Products Corporation
102 Old Mill Road SE
Cartersville, GA 30120

Salmonella Preincubation Mutagenicity Assay

FINAL REPORT

Sponsor: Chemical Products Corporation
102 Old Mill Road SE
Cartersville, GA 30120

Authorized Representative: Mr. Jerry Cook

Performing Laboratory: BioReliance
9630 Medical Center Drive
Rockville, Maryland 20850

Test Article I.D.: Quinone 3

Sponsor Project No.: 5893

BioReliance Study No.: AA22JL501004.BTL

Test Article Description: tan powder

Storage Conditions: room temperature; protected from exposure to light and moisture

Test Article Receipt: 05 October 99

Study Initiation: 20 October 99

Study Director: *Valentine O. Wagner III* 22 Dec 1999
Valentine O. Wagner III, M.S. Date

EXPERIMENTAL DESIGN AND METHODOLOGY

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975). Tester strain TA98 is reverted from auxotrophy to prototrophy by frameshift mutagens. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Experimental Design

The test system was exposed to the test article via the preincubation methodology described by Yahagi *et al.* (1977). The test article was tested at a minimum of eight dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate.

Plating and Scoring Procedures

In the preincubation method, one-half (0.5) milliliter of S9 or sham mix, 100 μ L of tester strain and 50 μ L of vehicle or test article were added to 13 X 100 mm glass culture tubes pre-heated to $37 \pm 2^\circ\text{C}$. After vortexing, these mixtures were incubated with shaking for 60 ± 2 minutes at $37 \pm 2^\circ\text{C}$. Following the preincubation, 2.0 mL of selective top agar was added to each tube and the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50 μ L aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at $37 \pm 2^\circ\text{C}$. Plates that were not counted immediately following the incubation period were stored at $2-8^\circ\text{C}$ until colony counting could be completed.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Evaluation of Results

For the test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of either one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA98 and TA100 were considered positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

RESULTS AND DISCUSSION

Solubility

Dimethyl sulfoxide was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells. Concentrations from 0.20 to 50 mg/mL were delivered to the test system as workable suspensions.

Mutagenicity Assay

The results of the mutagenicity assay are presented in Tables 1 through 4 and summarized in Table 5. These data were generated in Experiments B1 and B2. Precipitate was observed at $\geq 250 \mu\text{g}$ per plate but no appreciable toxicity was observed.

In Experiment B1, no positive responses were observed with tester strains TA98 and TA100 in the presence of S9 activation and with tester strain TA98 in the absence of S9 activation. Due to an unacceptable positive control value, tester strain TA100 in the absence of S9 activation was not evaluated but was retested in Experiment B2.

In Experiment B2, no positive response was observed with tester strain TA100 in the absence of S9 activation.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the *Salmonella* Preincubation Mutagenicity Assay indicate that under the conditions of this study, **Quinone 3** did not cause a positive response with either of the tester strains in the presence and absence of Aroclor-induced rat liver S9.

Salmonella Mutagenicity Assay

Table 1

Test Article Id : Quinone 3
 Study Number : AA22JL 301004 BTL
 Strain : TA98
 Liver Microsomes : None
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 10 µL
 Experiment No : 81
 Cells Seeded : 2.6 X 10⁶
 Date Plated : 10/26/99
 Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code*	Average Revertants	Standard Deviation
Vehicle	01	8	L	19	6
	02	12	L		
10	01	9	L	19	6
	02	8	L		
20	01	7	L	19	6
	02	7	L		
40	01	7	L	19	6
	02	7	L		
80	01	11	L	19	6
	02	10	L		
160	01	12	INP	19	6
	02	11	INP		
320	01	13	INP	19	6
	02	10	INP		
640	01	8	INP	19	6
	02	7	INP		
1280	01	7	INP	19	6
	02	7	INP		
Percent revertants per 10 ⁶ cells per µg			Percent revertants per 10 ⁶ cells per µg		
	01	11.25	L	19	6
	02	12.00	L	19	6

*Background codes are defined as follows: L = low background; M = medium background; H = high background; INP = inverted plate

Salmonella Mutagenicity Assay

Table 2

Test Article Id : Quinone 3
 Study Number : AA22JL501004.BTL Experiment No : B1
 Strain : TA98 Cells Seeded : 2.5×10^8
 Liver Microsomes : Rat liver S9 Date Plated : 10/26/99
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 50 μ L Counted by : hand

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	13	1	18	7
	02	23	1		
10	01	14	1	20	8
	02	26	1		
25	01	13	1	14	4
	02	12	1		
50	01	25	1	11	5
	02	12	1		
100	01	26	1	11	5
	02	20	1		
250	01	10	INF	11	5
	02	14	INF		
500	01	20	INF	11	5
	02	21	INF		
1000	01	25	INF	11	5
	02	20	INF		
2500	01	20	INF	11	5
	02	21	INF		

POSITIVE CONTROL - AMINO DIPHENYL AMINE (100 μ g per plate)
 1 20 1
 2 20 1

Background mutation rate of TA98 strain was 1.7 x 10⁻⁸ revertants per cell per plate per generation. The background mutation rate of TA98 strain was 1.7 x 10⁻⁸ revertants per cell per plate per generation. The background mutation rate of TA98 strain was 1.7 x 10⁻⁸ revertants per cell per plate per generation. The background mutation rate of TA98 strain was 1.7 x 10⁻⁸ revertants per cell per plate per generation.

Salmonella Mutagenicity Assay

Table 1

Test Article Id: Quinone
 Study Number: AA2251 3-1000 STL Experiment No: 52
 Strain: TA100 Cells Seeded: 0.8×10^8
 Liver Microsomes: None Date Plated: 11/23/99
 Vehicle: dimethyl sulfoxide (DMSO)
 Plating Aliquot: 50 μ L Counted by: machine

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
0.01	01	0	1	0	0
	02	0	1		
	03	0	1		
	04	0	1		
25	01	0	1	0	0
	02	0	1		
50	01	28	1	13	1
	02	24	1		
100	01	0	1	0	0
	02	0	1		
250	01	0	NP	0	0
	02	0	1		
500	01	0	NP	0	0
	02	0	NP		
1000	01	0	NP	0	0
	02	0	NP		

Revertants per plate = (total revertants) / (number of plates) = 100 / 10 = 10

Standard deviation = $\sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$

NP = No Plaque

Salmonella Mutagenicity Assay

Table 4

Test Article Id : Quinone 3
 Study Number : AA22JL501004.BTL Experiment No : B1
 Strain : TA100 Cells Seeded : 0.9×10^8
 Liver Microsomes : Rat liver S9 Date Plated : 10/26/99
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 50 μ L Counted by : machine

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	146	1	143	5
	02	139	1		
10	01	102	1	102	15
	02	123	1		
25	01	184	1	166	54
	02	107	1		
50	01	129	1	138	12
	02	146	1		
100	01	124	1	138	8
	02	132	1		
250	01	147	INF	131	12
	02	115	INF		
500	01	113	INF	113	5
	02	129	INF		
1000	01	167	INF	142	12
	02	114	INF		
2500	01	193	INF	171	14
	02	123	INF		

Positive Control: 1001 2,4-Diaminodiphenylamine 100 μ g/plate (1000)

01 137
 02 127

Background: 1001

1001 1001

1001 1001
 1001 1001
 1001 1001

Salmonella Mutagenicity Assay
 Summary of Results

Table 5

Test Article ID	Quinone	BTL		Experiment Nos	B1/B2
Study Number	AA2202	54/05	54/06		
Average Rate (1/10 ⁸) for Plate + Standard Deviation					
Liver Microsomes - None					
Dose (ug)	TA98	TA100			
0.0	19 ± 6	137 ± 5	5		
10	9 ± 1	178 ± 18	18		
25	11 ± 1	207 ± 19	19		
50	11 ± 1	151 ± 11	17		
100	11 ± 1	171 ± 11	17		
200	11 ± 1	171 ± 11	17		
400	11 ± 1	171 ± 11	17		
1000	11 ± 1	171 ± 11	17		
2500	11 ± 1	171 ± 11	17		
5000	11 ± 1	171 ± 11	17		
Pos	100 ± 0	100 ± 0	100		
Liver Microsomes - Rat Liver					
Dose (ug)	TA98	TA100			
0.0	8 ± 1	147 ± 10	10		
10	11 ± 1	171 ± 11	17		
25	11 ± 1	171 ± 11	17		
50	11 ± 1	171 ± 11	17		
100	11 ± 1	171 ± 11	17		
200	11 ± 1	171 ± 11	17		
400	11 ± 1	171 ± 11	17		
1000	11 ± 1	171 ± 11	17		
2500	11 ± 1	171 ± 11	17		
5000	11 ± 1	171 ± 11	17		
Pos	100 ± 0	100 ± 0	100		

FINAL REPORT

Study Title

Salmonella Preincubation Mutagenicity Assay

Test Article

Quinone 4

*Purified
Reagent Grade
Anthraquinone*

Sponsor Project Number

5893

Authors

Valentine O. Wagner, III, M.S.
Sean M. Caruthers, B.S.

Study Completion Date

December 22, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

AA22JM 501004 R11

Sponsor

Chemical Products Corporation
102 Old Mill Road
Carmichael, CA 95612

EXPERIMENTAL DESIGN AND METHODOLOGY

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975). Tester strain TA98 is reverted from auxotrophy to prototrophy by frameshift mutagens. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Experimental Design

The test system was exposed to the test article via the preincubation methodology described by Yahagi *et al.* (1977). The test article was tested at a minimum of eight dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate.

Plating and Scoring Procedures

In the preincubation method, one-half (0.5) milliliter of S9 or sham mix, 100 μ L of tester strain and 50 μ L of vehicle or test article were added to 15 X 100 mm glass culture tubes pre-heated to $37 \pm 2^\circ\text{C}$. After vortexing, these mixtures were incubated with shaking for 60 ± 2 minutes at $37 \pm 2^\circ\text{C}$. Following the preincubation, 24 mL of selective top agar was added to each tube and the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50 μ L aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at $37 \pm 2^\circ\text{C}$. Plates that were not counted immediately following the incubation period were stored at $2-8^\circ\text{C}$ until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Revertant colonies for a given tester strain and activation condition (except for positive controls) were counted either entirely by automated colony counter or entirely by hand using the plate counter facility. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Evaluation of Results

For the test article to be evaluated positive it must cause a dose-related increase in the mean revertants per plate of either one or both tester strains at a minimum of two increasing concentrations of test article. Data sets for tester strains TA98 and TA100 were judged positive if there is a dose-related increase in mean revertants per plate of one or both equal to or greater than two times the mean revertants per plate of the vehicle control.

RESULTS AND DISCUSSION

Solubility

Dimethyl sulfoxide was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells. The test article was soluble in dimethyl sulfoxide at a maximum concentration of approximately 1.0 mg/mL. Concentrations from 2.0 to 50 mg/mL were delivered to the test system as workable suspensions.

Mutagenicity Assay

The results of the mutagenicity assay are presented in Tables 1 through 4 and summarized in Table 5. These data were generated in Experiments B1 and B2. Premutation was observed at ≥ 25 μ g per plate but no appreciable toxicity was observed.

In Experiment B1, no positive responses were observed with tester strains TA98 and TA100 in the presence of S9 activation and with tester strain TA98 in the absence of S9 activation. Due to an unacceptable positive control value, tester strain TA100 in the absence of S9 activation was not evaluated but was retested in Experiment B2.

In Experiment B2, no positive response was observed with tester strain TA100 in the absence of S9 activation.

CONCLUSION

All critical to a valid study were met as described in the protocol. The results of the *Salmonella* Preincubation Mutagenicity Assay indicate that under the conditions of this study, **Quinone 4** did not cause a positive response with either of the tester strains in the presence and absence of Aroclor induced rat liver S9.

Salmonella Mutagenicity Assay

Table 2

Test Article Id	Quinone		Experiment No	B1
Study Number	AA22EM-01-004-BTL		Cells Seeded	2.6×10^8
Strain	TA98		Date Plated	10/26/09
Liver Microsomes	Rat Liver S9		Counted by	hand
Vehicle	dimethyl sulfoxide (DMSO)			
Plating Aliquot	50 μ L			

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	13	1	18	5
	02	23	1		
10	01	17	1	17	5
	02	11	1		
25	01	17	1	17	5
	02	17	1		
50	01	14	1	14	10
	02	18	1		
100	01	7	1	7	10
	02	7	1		
500	01	15	INF	15	10
	02	17	INF		
1000	01	14	INF	14	10
	02	17	INF		
2000	01	14	INF	14	10
	02	17	INF		

Revertants per plate = (total revertants - background) / (number of plates - background plates) = (13 - 1) / (2 - 1) = 12

Salmonella Mutagenicity Assay

Table 3

Test Article Id : Quinone 4
 Study Number : AA22JM.501004.BTL Experiment No 82
 Strain : TA100 Cells Seeded : 0.8×10^8
 Liver Microsomes : None Date Plated : 11/23/99
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 50 μ L Counted by machine

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	190	1	187	5
	02	183	1		
10	01	166	1	172	5
	02	178	1		
25	01	179	1	179	5
	02	179	1		
50	01	190	1	187	5
	02	183	1		
100	01	193	1	193	5
	02	197	1		
250	01	179	INF	184	15
	02	200	INF		
500	01	184	INF	187	15
	02	197	INF		
1000	01	179	INF	184	15
	02	197	INF		
2500	01	178	INF	184	15
	02	183	INF		

Positive Control, sodium azide 10 μ g per plate

01	217	1
02	212	1

^aBackground factor for calculation of

NSD^b

^bNSD = (Revertants - Background) /

Plating Aliquot

Salmonella Mutagenicity Assay

Table 4

Test Article Id : Quinone 4
 Study Number : AA22JM 501004 BTL Experiment No : B1
 Strain : TA100 Cells Seeded : 0.9×10^8
 Liver Microsomes : Rat liver S9 Date Plated : 10/26/99
 Vehicle : dimethyl sulfoxide (DMSO)
 Plating Aliquot : 50 μ L Counted by : machine

Concentration μ g per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	139	L	143	8
	02	139	L		
10	01	139	L	140	11
	02	139	L		
25	01	121	L	125	12
	02	121	L		
50	01	110	L	114	23
	02	118	L		
100	01	129	L	126	9
	02	129	L		
250	01	124	INP	124	16
	02	101	INP		
500	01	119	INP	119	11
	02	119	INP		
1000	01	116	INP	116	11
	02	111	INP		
1500	01	101	INP	101	11
	02	101	INP		
Percentile Control	01	139	L	139	8
	02	139	L		

Abbreviations:

L: Number of revertants per plate
 INP: Inconclusive Plate
 S: Significant increase in revertants
 NS: Not Significant increase in revertants

INP: Inconclusive Plate
 S: Significant increase in revertants
 NS: Not Significant increase in revertants

Salmonella Mutagenicity Assay
Summary of Results

Table 5

Test Article Id : Quinone 4
Study Number : AA22JM 501004.BTL Experiment Nos 81/82

Average Revertants Per Plate ± Standard Deviation
Liver Microsomes: None

Dose (µg)	TA98	TA100*
0.0	19 ± 6	187 ± 5
10	18 ± 5	172 ± 8
25	11 ± 7	171 ± 3
50	12 ± 9	187 ± 5
100	12 ± 1	195 ± 3
250	14 ± 1	190 ± 15
500	12 ± 5	196 ± 16
1000	9 ± 2	179 ± 9
2500	15 ± 4	191 ± 10
Pos	626 ± 37	621 ± 5

Liver Microsomes: Rat Liver S⁹

Dose (µg)	TA98	TA100*
0.0	18 ± 7	143 ± 5
10	12 ± 1	117 ± 12
25	17 ± 1	115 ± 20
50	21 ± 10	114 ± 23
100	26 ± 8	126 ± 9
250	14 ± 3	112 ± 16
500	14 ± 1	119 ± 1
1000	14 ± 1	114 ± 16
2500	17 ± 4	89 ± 17
Pos	840 ± 35	435 ± 28

* TA100 is a histidine-requiring strain of Salmonella typhimurium. The asterisk (*) indicates that the revertants are histidine-requiring.

CERTIFICATE OF AUTHENTICITY

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