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July 25, 2000

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(Attn: FYI Coordinator)
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U.S. Environmental Protection Agency
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Subject: Anthraquinone (CAS # 84-65-1); FYI submission concerning NTP studies

Dear Sir or Madam:

The attached letter has been sent to the California EPA's Office of Environmental Health Hazard Assessment in response to their June 2, 2000 Request for Relevant Information concerning "Chemicals Under Consideration for Possible Listing via the Authoritative Bodies Mechanisms" of Proposition 65. Anthraquinone (CAS # 84-65-1) is one of the chemicals under consideration for possible listing; the consideration of Anthraquinone is due solely to NTP draft Technical Report 494.

The attached letter describes factual errors in draft Technical Report 494; NTP has been informed of these errors and of the likelyhood that the results of the 2-year studies with Anthraquinone were confounded by the presence of a mutagenic impurity, 9-nitroanthracene, found in the Anthraquinone employed by NTP for the studies.



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Sincerely,

Jerry Cook

Jerry A. Cook
Technical Director

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July 17, 2000

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Ms. Cynthia Oshita
Office of Environmental Health Hazard Assessment
301 Capitol Mall, 2nd Floor
Sacramento, California 95814

Subject: Information demonstrating that Anthraquinone (CAS # 84-65-1) should not be listed via the authoritative bodies mechanisms

Dear Ms. Oshita;

This letter is in response to the June 2, 2000 "Request for Relevant Information" concerning Chemicals Under Consideration for Possible Listing via the Authoritative Bodies Mechanisms. It presents information demonstrating that the criteria for listing chemicals through the "authoritative bodies" mechanisms as set forth in 22 CCR Section 12306 are not met in the case of Anthraquinone (CAS # 84-65-1).

It is being submitted by Chemical Products Technologies, LLP, an importer of Anthraquinone into the United States; Chemical Products Technologies has been assisted in evaluating Anthraquinone toxicological information by Chemical Products Corporation, a Georgia corporation which has an interest in Anthraquinone.

Long-term studies conducted by the National Toxicology Program (NTP) found that the AQ powder employed in the study produced clear evidence of carcinogenic activity in female F344/N rats based on increased incidences of renal tubule neoplasms,

and clear evidence of carcinogenic activity in male and female B6C3F₁ mice based on increased incidences of liver neoplasms. These studies have been reported in NTP draft technical report number 494 (NTP494); this draft document has led to the consideration of Anthraquinone (AQ) for possible listing through California's "authoritative bodies" mechanisms.

This letter contains information demonstrating that the compound AQ is not a mutagen, and that it is likely that the carcinogenic activity observed in NTP494 was the result of a genotoxic contaminant present in the sample of AQ powder tested, rather than the AQ itself. The pattern of induced tumors in NTP494 is consistent with a direct acting mutagen, rather than a nongenotoxic carcinogen. A sample of the AQ employed by NTP for the studies reported in NTP494 was obtained from NTP and subjected to detailed chemical analysis; 9-nitroanthracene was found in the sample. The IARC Summary for this compound states, "9-nitroanthracene was mutagenic to *Salmonella typhimurium* in the presence and absence of an exogenous metabolic system."

Anthraquinone (9,10-anthracenedione) is used as a raw material for the production of dyes and pigments, and as a catalyst in the Kraft Process for the production of paper. Although it is no longer produced in the United States, 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. In a second production method, benzene and phthalic anhydride undergo the Friedel-Crafts reaction to yield o-benzoylbenzoic acid, which is contacted 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

In a third production method, a Diels-Adler synthesis from 1,4-naphthoquinone and 1,3-butadiene is being employed in Japan to produce AQ. A fourth possible production method based on styrene is reportedly being practiced in Eastern Europe; this technology was developed by BASF Corp. in the 1970's. The final commercial AQ products from these different production processes vary in color and impurities contents.

While the great majority of the technical-grade AQ commercially available in the United States is the tan-colored AQ 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 AQ sample employed by NTP in the studies is described in NTP494 as a golden yellow powder.

A large number of mutagenicity assays have reported that AQ is not genotoxic. Negative results in the Ames salmonella bacterial mutagenicity assay have been reported six times in published reports and once in a TSCA submission [Salamone et al., 1979; Brown and Brown, 1976; Gibson et al., 1978; Anderson and Styles, 1978; Tikkanen et al., 1983; Sakai et al., 1985; TSCA OTS 0521344]. Four samples of AQ were submitted for mutagenicity testing by CPC, three of the four yielded negative results (BioReliance, 1999).

In contrast to the many reports of a lack of AQ mutagenic activity as noted above, there are three reports of individual samples of AQ being mutagenic in the Ames salmonella mutagenicity assay. The pattern of activity of AQ reported in all three cases is, however, unusual in that mutagenic activity was seen without metabolic activation and addition of an S-9 metabolic activation system reduced or eliminated the response [BioReliance, Quinone 1, 1999; Liberman et al., 1982; Zeiger et al., 1988]. This suggests

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that a mutagenic contaminant was present in the positive Ames test samples that was either directly mutagenic or could be activated by bacterial metabolism. The presence of a mutagenic contaminant in an AQ sample being responsible for a positive mutagenicity response is actually demonstrated in a TSCA submission included with this letter and discussed below.

NTP did not subject the AQ sample employed in NTP494 to mutagenicity testing; an earlier sample had been tested by NTP (Zeiger et al., 1988). The statement in NTP494 that AQ is mutagenic in *Salmonella typhimurium* strains TA 98 and TA 100 with and without S9 activation enzymes is based on that earlier report; no other study has ever confirmed the Zeiger et al. (1988) findings.

A portion of the retained NTP494 AQ sample was obtained from NTP; this golden yellow powder bore a label stating, "Anthraquinone, Battelle Task Identifier: 4-064-SHIP-179, lot: 5893, CAS: 84-65-1, Store: RT". The NTP494 AQ sample and three additional AQ samples were submitted to BioReliance, Inc. (formerly Microbiology Associates, Inc., the laboratory employed by NTP for the Zeiger et al. testing) for mutagenicity testing in *Salmonella typhimurium* strains TA98 and TA100. The samples were submitted to BioReliance, Inc., labeled only as "Quinone 1", "Quinone 2", "Quinone 3", and "Quinone 4". These four samples have been subjected to chemical analysis, and each was found to contain greater than 98% anthraquinone. Quinone 1 is the NTP494 sample. All available bacterial mutagenicity information for AQ, including the results for these four samples, is shown in Table 1.

Table I. Bacterial Mutagenicity Test Results Reported 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		NEG	
Anderson & Styles		NEG		NEG		NEG				NEG
Tikkanen et al.	NEG	NEG	NEG	NEG						
Sakai et al.	NEG	NEG	NEG	NEG						
Quinone 1 - NTP 494 sample	POS	POS	POS	NEG						
Quinone 2 - Friedel-Crafts AQ from China	NEG	NEG	NEG	NEG						
Quinone 3 - Friedel-Crafts AQ from China	NEG	NEG	NEG	NEG						
Quinone 4 - Purified reagent grade AQ	NEG	NEG	NEG	NEG						
TSCA Submission OTS 0521344										
Sample A*	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.

The only positive results in the BioReliance studies were observed with the NTP494 bioassay material, Quinone 1. All four study reports are included with this

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letter. The three additional samples of AQ submitted to BioReliance, Inc. for testing consisted of one golden yellow reagent grade sample of AQ subjected to further purification before submission (it was dissolved in concentrated sulfuric acid and subsequently precipitated by addition of hot water) to yield a pale yellow powder (Quinone 4), as well as two different tan-colored AQ samples produced in China by the Friedel-Crafts reaction (Quinone 2 and Quinone 3).

A TSCA submission from the 1970's reports the results of mutagenicity testing on 6 samples of AQ produced by various production processes. Only one of the six AQ samples showed mutagenic activity in the Ames test; a mutagenic contaminant was subsequently found in the AQ sample yielding the positive result. When the contaminant, 0.15% nitroanthracene, was removed and the sample was retested, the purified AQ sample was no longer mutagenic. Thus, this report is not an indication that AQ is mutagenic, but rather that small concentrations of contaminants in AQ can produce positive mutagenic test results.

The information in the TSCA submissions about a contaminated sample of AQ being the only one of six AQ samples to test positive for mutagenicity is not addressed in the NTP494; there is no indication that the NTP researchers were aware that this information existed.

The two published studies reporting that AQ is mutagenic (Lieberman et al., 1982; Zeiger et al., 1988) both obtained their test material from Aldrich Chemical Company, although at different times. Zeiger et al. (1988) is the only previous report of mutagenicity in strain TA100; only TA98 and TA100 were tested in this study. Lieberman et al. (1982) only found mutagenic activity in the absence of S9 activation

enzymes in strains TA98, TA1537, and TA1538; he found no mutagenic activity in TA100. NTP494 incorrectly states on page 23, "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. (Liberman et al., 1982; Zeiger et al., 1988)."

NTP494 reports that decreased incidences of mononuclear cell leukemia in male and female rats were attributed to exposure to anthraquinone. NTP494 also reports negative results from an acute exposure mouse bone marrow test with anthraquinone administered by interaperitoneal injection.

NTP494 reports that significant increases in the frequencies of micronucleated normochromatic erythrocytes were observed in peripheral blood samples from male and female mice exposed to AQ in feed for 14 weeks; however, an examination of the data calls this conclusion into question. The study scientist, Dr. Richard Irwin, has been informed that a review of the data contained in NTP494 led an expert in this area to the conclusion that since only the highest dose female group differed significantly from the control frequency by pairwise comparison, this does not represent a positive result.

22 CCR Section 12306 subsection (f) states, "The lead agency shall find that a chemical does not satisfy the definition of "as causing cancer" if scientifically valid data which were not considered by the authoritative body clearly establish that the chemical does not satisfy the criteria of subsection (e), paragraph (1) or subsection (e), paragraph (2)." We believe that the information contained in this letter establishes that the pure chemical AQ is not a bacterial mutagen, and that the mutagenicity found upon testing of the NTP494 sample of AQ demonstrates that that particular sample is sufficiently

contaminated with 9-nitroanthracene, a genotoxic compound, to confound the results of the bioassay conducted by NTP. Therefore, we submit that the criteria of subsection (e), paragraph (2) are not met in the case of Anthraquinone.

A sample of the NTP494 AQ was obtained by Gibson et al. (1997) and employed for additional testing. Syrian hamster embryo (SHE) cell transformation results were negative; however, when subjected to the SHE cell *in vitro* micronucleus assay, the NTP494 AQ sample did induce a significant increase in the percentage of micronucleated binucleated cells (6.6% compared to 3.2% in the control), but only at the highest concentration tested, 25 µg/mL. This report is incorrectly characterized in NTP494 on page 23 as, "dose-related increases in micronuclei were reported in cultured Syrian hamster embryo cells treated with 3.13 to 25 µg/mL anthraquinone".

An AQ sample obtained commercially was tested for induction of forward mutations in cultured human BT lymphoblastoid cells that constitutively express cytochrome P4501A1, which is known to be necessary for the metabolism of many promutagens (Durant et al., 1996). This is a metabolically competent cell line for polycyclic aromatic compounds, and this test involves a relatively long duration of treatment. AQ exhibited no mutagenic activity; 9-nitroanthracene was also tested and found to be mutagenic.

An 18 month bioassay conducted with AQ obtained from a commercial source and administered to male and female mice of two F₁ hybrid stocks reported no significant indication of tumorigenicity after oral administration [Innes et al., 1969].

NTP494 reports that several metabolites of AQ were detected in rat urine; 1-hydroxyanthraquinone and 2-hydroxyanthraquinone are the only compounds named. The

compound 1-hydroxyanthraquinone is present in certain medicinal plants such as *Rubia tinctorum* L. and is a rodent colon carcinogen. Tanaka et al. (2000) reports that the polymerase chain reaction-single strand conformation polymorphism analysis revealed that no mutations in *Ki-ras* and *p53* (and, additionally, no mutations of APC) were found in rat colonic neoplasms induced by 1-hydroxyanthraquinone. Tikkanen et al. (1982) tested 2-hydroxyanthraquinone obtained from *Morinda umbellata* in TA98, TA100, and TA2637; no mutagenic activity was found in TA98 and mutagenicity was found in TA100 and TA2637 only in the presence of rat S9 (the number of revertants in TA100 with S9 mix was about two-fold increase).

Genotoxic carcinogens tend to induce cancer in multiple target organs, in both sexes, and across species [Ashby et al., 1991]. This was the pattern produced by the NTP494 AQ sample 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 [Butterworth et al., 1995; Ashby et al., 1991]. The fact that mutagenic activity of the NTP494 AQ sample was readily measured, indicates that the impurity must be a fairly potent mutagen.

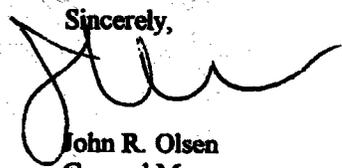
Both NTP and the EPA TSCA Submissions Desk have been provided the information contained in this letter. A review of the analytical data retained by NTP has shown that the existence an impurity was detected in the initial analysis of the NTP494 AQ sample, but the impurity was not identified.

Copies of the mutagenicity references, including the TSCA submission, are included with this letter. If we can answer any questions concerning this letter or supply

any further information relating to this matter, please telephone Jerry Cook at Chemical Products Corporation, telephone number 770-382-2144.

In summary, this letter contains information demonstrating that AQ has been repeatedly tested and found not to be mutagenic. NTP494 incorrectly characterizes AQ as being a mutagen and develops a carcinogenicity model based on this incorrect characterization. It is likely that the tumorigenic activity reported in NTP494 was the result of a genotoxic contaminant, 9-nitroanthracene, present in the sample of AQ powder tested by NTP. We believe that we have demonstrated that the criteria for listing chemicals through the "authoritative bodies" mechanism as set forth in 22 CCR Section 12306 are not met in the case of Anthraquinone (AQ), and we respectfully request that AQ not be listed.

Sincerely,



John R. Olsen
General Manager

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* These documents are included with this letter.

CERTIFICATE OF AUTHENTICITY

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