AIREPEL
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January 18, 2005

National Institutes of Health
Associate Director for Communications
Office of the Director
Building 1, Room 344
9000 Rockville Pike
Bethesda, MD 20892

Subject: Request for Correction of Information

Dear Madam or Sir:

This request of information is submitted by Airepel Humane Bird Management Services (Airepel) pursuant to Section 515(a) of the Treasury and General Government Appropriations Act for Fiscal Year 2001[1] (the Information Quality Act) and the implementing guidelines issued by the OMB[2], the National Institutes of Health[3] and the U.S. Department of Health and Human Services[4]. The National Toxicology Program (NTP) as an agency of the HHS is subject to the guidelines collectively promulgated by the OMB, NIH and HHS.

Airepel is a wholly owned subsidiary of Arkion Life Sciences LLC of Wilmington, Delaware. Airepel runs a bird management business that uses anthraquinone (AQ), as a humane bird repellent registered as a biopesticide by the USEPA. Airepel is also the patent holder for the uses of AQ in this field of endeavor.

Airepel seeks a correction of information disseminated by the NTP within the stated guidelines.

1. Correction of the NTP study TR 494[5] to resolve the conflicted science as reviewed in discrepancy resolution document[6].
2. Correction of the NTP study TR 494 to include the peer reviewed literature specific to the study[7].

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[1] 44 U.S.C. 3516 note.
[2] *Guidelines for Ensuring and Maximizing the Quality, Objectivity, Utility and Integrity of Information by Federal Agencies*, 67 Fed. Reg. 8452 (Feb. 22, 2002), available at www.whitehouse.gov/omb/fedreg/reproducible2.pdf.
[3] *Guidelines for Ensuring Quality of Information Dissemination to the Public*, available at http://www.hhs.gov/infoquality.
[4] *Guidelines for Ensuring Quality of Information Dissemination to the Public,* See also MEMORANDUM FOR PRESIDENT'S MANAGEMENT COUNCIL (http://www.whitehouse.gov/omb/inforeg/pmc_graham_100402.pdf) From John D. Graham. Re: Agency Draft Information Quality Guidelines (June 10, 2002)
[5] Toxicology and Carcinogenesis Studies of Anthraquinone (CAS No. 84-65-1) in F344/N Rats and B6C3F1 Mice (Feed Studies) NTP TR 494 NIH Publication No. 04-3953
[6] Attachment A "Study Discrepancy Resolution".

3. Correction of the NTP study TR 494 abstract to reflect the changes in the body of the text.
4. Correction of the Title of the NTP study 494 to reflect the actual nature of the sample tested to read "Toxicology and Carcinogenesis Studies of Mutagen Contaminated Anthraquinone (CAS No. 84-65-1) in F344/N Rats and B6C3F_1 Mice (Feed Studies)".
5. Correction of all related web site postings of the current text and abstract of the study.
6. Explanation by the NTP of the apparent non-homogeneity of lot 5893 used in the bioassay.
7. 40 CFR Part 792.105 (a) requires the method of synthesis of a test article to be documented for each sample of a test article. NTP should follow these guidelines and account for the contaminants that result.

Airepel is aware of the pending approved changes to the study based on a peer review meeting held December 9, 2004. The final decisions made at the public meeting did not address the peer-reviewed science presented to the NTP. Data shown to the review committee three days prior to the meeting further confounds the conclusions of the study. This petition is filed to give the NTP notice of the corrections to the study that would preserve the high quality of the scientific approach that has been the hallmark of the agency prior to final publication.

NIH's Guidelines require that a petition for correction contain several substantive components. These include a description of the specific material that is proposed for correction, the reasons why the disputed information does not comply with the OMB or NIH Guidelines and is in error, an explanation of how the petitioning party is affected by the error, and suggested recommendations for what corrective actions should be taken.

How the petitioner is affected.

Airepel is adversely affected by the current study language in contracts related to the use of AQ in the form of Flight Control® Plus that specifically refer to the outcome of the NTP decision to name AQ as a mutagen and as a carcinogen. The impact of the reversal of a previous decision to retitle the study is causing Airepel substantial economic loss. Making the requested corrections would alleviate this damage.

Study TR-494 labels anthraquinone as a mutagen and a carcinogen. This identification affects the previously issued EPA biopesticide use label for AQ as a non-toxic bird repellent listed as not mutagenic. The use of the product is the principle business of the petitioner and EPA decisions affect the viability of the product and the business. The intent of Airepel is to offer nontoxic alternatives to controlling birds and our science has indicated that AQ is not a mutagen and this information is in conflict with the NTP study.

Background to the Scientific Discrepancies in TR 494

A full review of the confounding science is included for reference as written by Dr. Byron Butterworth in Attachment C. The National Toxicology Program has peer reviewed the Anthraquinone study TR 494 over the last six years. The peer review process has been complicated by the identification

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[7] *"The preparation of anthraquinone used in the National Toxicology Program cancer bioassay was contaminated with the mutagen 9-nitroanthracene"* Byron E. Butterworth et al.,**Mutagenesis** vol.16 no. 2 pp.169-177, 2001, also found as Attachment B. "*Contamination is a Frequent Confounding Factor in Toxicology Studies with Anthraquinone and Related Compounds*", Byron Butterworth et.al, **International Journal of Toxicology**, 23:335-344, 2004 also found in Attachment B.

of contaminates in the test article that may have influenced the outcome of the study. It is our contention that these contaminates were biologically active in very low concentrations and could have explained the organ damage reported in the bioassay. Our data is available in two peer-reviewed studies attached to this letter in Attachment B. In February of this year, the peer review committee recommended a solution by retitling the study to identify the source of the AQ. This solution was acceptable and correctly identified the test article under study. On December 9th, the committee reconvened and reversed this decision based on new data offered on the web site three days prior to the meeting.

The NTP presented results of an Ames study that retested the study sample plus three other AQ samples. The data was offered to the review committee as proof that alternate AQ sources were also mutagenic in Ames testing and therefor the original title would be accurate. The results were offered without any normal test information and the results were presented as conclusive evidence that Diels Alder AQ was positive by Ames testing. The test was unavailable for review. The results confound rather than elucidate the debate. The test results were not evaluated in light of previously reported peer-reviewed results.

There should be no doubt that AQ is not mutagenic in Ames testing when it lacks mutagenic contaminants. Furthermore the fact that NTP repeated the initial mutagenic work on the test article designated lot 5893 that contained the contamination and found the results to be negative throws one of the basic tenants of the study into question. Anthraquinone is labeled a mutagen in the abstract and throughout the body of the text. This designation must now be removed and statements such as "**anthraquinone and most substituted anthraquinones are mutagens. Therefore it is not surprising that many anthraquinones have been carcinogenic in long-term animal studies." (TR 494 draft p.113)** must be revised to reflect the actual results. Recommendations are made on a point-by-point basis in Attachment A.

The new data presented on December 9th is indicative of the confusion that exists with study 494 and we request a full record of the study since it was presented to the review committee as conclusive in proving that Ames testing can show that alternative sources of AQ are mutagenic.

Table 1
NTP data presented Dec. 9, 2004

Testing Laboratory	Preparation	Percent Impurities	Conc. (μg/plate)	Rev./plate in strain TA98 *minus* S9
NTP	Anthracene derived AQ Lot # 5893	3% (TR 494 1999)	0 333 1,000 2,500	15 225 723 1,497
NTP	Anthracene derived AQ Lot # 5893 **PURIFIED**	0% (TR 494 2004)	0 1,000 3,333 10,000	12 13 12 12
Arkion	Anthracene derived AQ Lot # 5893	0.65% (Butterworth Et al. 2001 & 2004)	0 500 1,000 2,000	18 116 213 433
Arkion	Anthracene derived AQ Lot # 5893 **PURIFIED**	0% (Butterworth Et al. 2001 & 2004)	0 500 1,000 2,000	14 11 15 22
NTP	Anthracene derived AQ Lot # 5893	0.15% NTP 12/09/04 meeting results only	0 1,000 3,000 10,000	7 4 6 6

The new data also showed that a Diels Alder AQ sample from Kawasaki Kasei tested positive. Since that source of AQ is identical to the AQ used in Airepel's work and has consistently been negative in all of our work (seven lots were negative and presented to USEPA for review in 1998), there is doubt about the results presented by the NTP. Combined with the discrepancies pointed out with sample 5893, Airepel will request a retain of the same lot number from Kawasaki and repeat the testing to clarify the confusion presented to the review committee. The outcome of these studies will be sent to the NTP and the OMB to document the confusion caused by the efforts made by the NTP to support the contention that AQ is mutagenic. Good science for good decisions requires no less.

Specific Nature of the corrections needed to the science in TR 494.

The essence of the scientific disagreement is the degree of influence caused by contamination of the test article and the role of the metabolites of anthraquinone in the outcome of the study. If contaminants can be removed by purification of the AQ and reverse the Ames test results then there must be a biologically significant influence. The alternate argument from the NTP claims that AQ metabolites and not the parent compound cause the damage. Data presented by the NTP showed that the two metabolites were 1 hydroxy and 2 hydroxy AQ. In a peer-reviewed analysis of this information[8], it was

[8] . "*Contamination is a Frequent Confounding Factor in Toxicology Studies with Anthraquinone and Related Compounds*", Byron Butterworth et.al, **International Journal of Toxicology**, 23:335-344, 2004

discovered that NTP was using another contaminated test substance in their analysis. The response from the NTP has been silence on this issue. If the metabolites are shown to be non-mutagenic the conclusions as stated are confounded and the mechanism of AQ activity is not clear. Documented testing shows how the potency of the identified contaminants in the study could have accounted for the damage to the rat tissue[9]. The science in question is covered in Attachment A "Discrepancy Resolution Document".

The coal tar derived AQ test material Lot 5893 provided by the NTP to Chemical Products Corporation and to Arkion Life Sciences exhibited mutagenic activity in the Ames test with similar magnitudes of response. In both cases, AQ purified from the test material was not mutagenic (Table 1). The NTP tested lot 5893 again and reported no mutagenic response without purification in the same study (Table 1). All testing done outside the NTP was done in duplicate by reputable commercial laboratories. The Chemical Products Corporation studies and the NTP studies were done using the pre-incubation protocol. The Arkion Life Sciences studies were done using the plate incorporation protocol.

A discrepancy was also found in the analytical studies of lot 5893. The NTP observed 0.15% contaminants for lot 5893 (presentation by Cynthia Smith at the NTP Technical Reports Subcommittee Review Meeting in Research Triangle Park, NC on December 9, 2004). Detailed studies conducted by Arkion for Lot 5893 reported a level of 0.65% contaminants.

"The most straightforward explanation of these apparently conflicting results is that the NTP test material was not homogeneous. Was lot 5893 from one large container? Was the material evenly mixed? Was Lot 5893 supplied in many smaller containers? What is the analytical and mutagenic consistency from one container to the next? Determination of substantial heterogeneity in the test material could invalidate the bioassay. Given that small differences in the contamination level can result in dramatically different mutagenic activity (Table 1). It is imperative that the NTP address this issue."[10]

40 CFR Part 792.105 (a) is entitled "Test, control and reference substance characterization". It states that the identity, strength, purity and composition, or other characteristics which will appropriately define the test, control or reference substances shall be determined for each batch and shall be documented before its use in a study. Methods of synthesis, fabrication, or derivation of the test, control or reference substance shall be documented by the sponsor or testing facility, and such location of documentation shall be specified." These requirements are standard for analytical laboratories that support toxicological and animal test studies. It is essential for the sponsor to provide information on identification of impurities and the concentrations of the impurities in the test substance that will influence the results so that the characterization work can be carried out in a timely and cost effective manner.

The only scientifically valid outcome of this confusion is to adopt the change in the title of the study to "Toxicology and Carcinogenesis Studies of Mutagen Contaminated Anthraquinone (CAS No. 84-65-1) in F344/N Rats and B6C3F1 Mice (Feed Studies)". A full reconciliation of the data presented in the attached studies in Attachment B should also be included in the abstract and in the body of the study to allow for accurate interpretation of the results reported in TR 494. With these changes, there would be no confusion as to the implications of the outcome of the efforts made by the NTP in conducting the bioassay.

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[9] *"The preparation of anthraquinone used in the National Toxicology Program cancer bioassay was contaminated with the mutagen 9-nitroanthracene"* Byron E. Butterworth et al.,**Mutagenesis** vol.16 no. 2 pp.169-177, 2001.

[10] Byron Butterworth personal communication, Attachment C

Our organization is willing to work closely with the team at NTP to provide test samples and to continue to help understand the true nature of anthraquinone. As Dr. Irwin states on p.17 of the draft study "the metabolism of anthraquinone is extremely complicated". There is no doubt that the test article caused significant damage in the rat study. The issue is clouded by the very basic question, what was tested.

Sincerely,

Ken Ballinger
Vice President
Airepel

Cc: John D. Graham, Administrator, OIRA, OMB

Attachment A

Discrepancy Resolution Document

TR –494 Draft Document Reference	Airepel Resolution Recommendation
p.13 Genetic Toxicity reference for **2-hydroxyanthraquinone as positive in strain TA98 without S9, negative in strains TA98 with S9 and TA100 with and without S9.**	Butterworth et al.[9] showed that **pure 2-hydroxyanthraquinone was negative in TA98 without S9** indicating a contaminated sample was employed by NTP. Without 2-OH AQ as a clear mutagen, the argument that 9-nitroanthracene would be a potential source of damage in the rat study is bolstered. NTP should modify this language to indicate discrepant results based on purity of the test article and reference the Butterworth paper at this point.
p.17 Dr. Irwin states in paragraph 2 the conclusions of the study showing **clear evidence of carcinogenicity in female rats and mice and some evidence in male rats.**	No reference is made to contamination of the test article clouding these conclusions. The uses of these terms are trigger words for the overall assessment of the molecule found on web sites and summary documents. Suggested modification would be; **results of this study need to be balanced with the potential influence of mutagenic contaminants found in the test article. No clear results can be made without this balanced view.**
p.27 References are made to the presence of 9-nitroanthracene contaminating other Ames tests done in the literature. Specifically Zeiger et al (1988).	No further mention is made of this clear confirmation of the issue in the abstract or conclusions of the study. Similar findings are found in Butterworth et al. (2001).
p.28 Third paragraph. **Thus, protocol characteristics, dose, and purity may well be important factors in the detection of mutagenicity of anthraquinone and substituted anthraquinone.**	Protocol characteristics did not yield any significant differences in analysis. The key differences were in purity of the test articles. This conclusion is not found in the abstract or conclusions of the draft. **The purity of the test article created biologically significant differences in the outcome of the mutagenicity testing.**
p.31 The purity of the test article varies by test method. **The HPLC/UV method shows 99.5% purity.**	No discussion about the impurities blinds the reader to the issue of mutagenic contamination. The results of the HPLC run by the NTP and the analytical work reported by Butterworth et al. (2001) are in close agreement. **Butterworth et al (2001) reports 0.6% contamination caused by 9-nitroanthracene (0.12%) and other nitroaromatics.**

p.93 **Anthraquinone (97% pure) (33 to 2,500 ug/plate) was mutagenic in *Salmonella typhimurium…Zeiger et.al, 1988.* Subsequent testing with 100% pure AQ … showed no mutagenic response…**	This reference had a contaminated AQ containing 0.12% 9-nitroanthracene as previously noted on p.27 that accounts for this positive result. This is a misleading statement as is the reference in table E1 connected to this statement. **The anthraquinone tested by Zeiger et al in 1988 gave confirmation to the testing done by Butterworth et al (2001) with the same contaminant at the same levels. Anthraquinone showed no mutagenic response in either study without the contamination of 9-nitroanthracene.**
p.93 There is a reference to the mutagenicity of 2-hydroxyanthraquione that starts with **2-Hydroxyanthraquinone (3.3 to 450 ug/plate) was mutagenic at low doses…**	Butterworth et al. (2004). Tested 2-OH AQ with and without contamination and found results from the Ames test dramatically different**. TA 98 with and without S9 results with pure 2-OH-AQ showed negative results and indicated no mutagenicity in Butterworth et al. (2004).**
p.113 Second paragraph. **In addition, anthraquinone and most substituted anthraquinones are mutagens. Therefore it is not surprising that many anthraquinones have been carcinogenic in long-term animal studies.**	These conclusive statements have proven false and need to be removed from the text. If other anthraquinones have been proven to be carcinogenic in animal studies, the relationship with 9,10 anthraquinone has to be made**. Pure AQ is not a mutagen in Ames testing. 2-OH AQ in its pure form as would be the case in a metabolic process is not a clear mutagen.** The basis of the paper rests on these statements.
p.114 Results shown in the table for Anthraquinone	The test molecule that gave these results included biologically active contaminants. There needs to be a reference to the contamination in all table references. This table will be used frequently in other publications and misleads the reader without a reference to the contamination and the composition of the contamination as stated by Zieger et al.(1988).
p.115 second paragraph. The defense of the testing done by the NTP vs. work done by Butterworth et al (2001) rests on two observations. The work done to confirm the mutagenicity of 9-nitroanthracene was not done on a bone fide sample and that all the contamination was a result of 9-nitroanthracene.	Butterworth et al (2001) simply purified the AQ sample used in the NTP bioassay and showed that mutagenicity was a function of contaminants not the AQ. 9-NA samples were identified and specified in the study and can be retested by any authority if this issue is key to the study. In the analytical evaluation of the NTP sample, additional contaminants were discovered and some were identified. More than one mutagen may have been active in the contaminants as stated in the

	study. This fact is not mentioned by the NTP.
P.116 third paragraph. **However, 2-hydroxyanthraquinone was mutagenic in TA98 in the absence of S9 and gave an equivocal response in the presence of S9.**	**Butterworth et al (2004) presents the results of the Ames test on purified 2-OH AQ as negative results with and without S9 in TA98. 2-OH AQ is not mutagenic**. This data removes the argument that 2-OH would overwhelm any response from 9-nitroanthracene in the study.
p.121 Table 23 calculates the relationship between 9-NA and 2-OH AQ as relative influences on the rat study based on the Ames results. Conclusions point to clear evidence of AQ as a carcinogen.	**Butterworth et al (2004) challenges the results for 2-OH AQ and implicates 9-nitroantracene as a potential contaminating influence in the rat study.** **The conclusions stating AQ had clear evidence does not address the contaminating influences that may have affected the results.**
Appendix E-3 Results. AQ (97% pure) (33 to 2,500 ug/plate) was mutagenic …**Subsequent testing with a 100% pure sample of anthraquinone (100 to 10,000 ug/plate) showed no detectable mutagenic response in TA98, TA100, or TA102, with or without 10% rat liver S9.**	Why are these results not discussed anywhere else in the study when the mutagenicity of AQ is mentioned? The effect of taking this result and placing it deep in an appendix without further comment insures the reader is unlikely to realize that the **NTP agrees with Butterworth et al. (2001) that purified AQ is not mutagenic in the Ames assay.**

Attachment B


NTP Mutagen Study.pdf


NTP Contamination.pdf

Attachment C

Concerns with the Purity and Homogeneity of the Anthraquinone (AQ) used in the National Toxicology Program (NTP) Bioassay

Author: Byron E. Butterworth

December 14, 2004

Background

In 1999 the NTP completed NTP TR 494 describing toxicology and carcinogenesis studies of anthraquinone (AQ) in F344/N rats and B6C3F_1 mice (NTP 1999). The NTP anthraquinone test material induced 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_1 mice (NTP, 1999).

The Problem with Coal Tar Derived AQ

The test material used in the NTP bioassay was Lot 5893 of AQ obtained from Zeneca Fine Chemicals. Unfortunately, this lot was coal tar derived AQ and contained biologically significant amounts of mutagenic impurities. At the time the bioassay was conducted, a common synthetic pathway to produce AQ was from the oxidation of anthracene. The anthracene used was distilled from coal tar and different lots of anthracene-derived AQ contained varying amounts of polycyclic aromatic hydrocarbon contaminants, particularly the mutagenic isomers of nitroanthracene (reviewed in Butterworth et al., 2001).

The Contamination Issue

The contamination problem with coal tar derived AQ was well known. Numerous published studies had shown that AQ was not a bacterial mutagen (reviewed in Butterworth et al., 2001). Despite this fact, the NTP TR 494 (NTP, 1999) reported that AQ in their hands was a potent bacterial mutagen (Table 1). This raised the concern that both the NTP mutagenicity test material as well as the NTP bioassay test material might have been contaminated. Mr. Jerry A. Cook of Chemical Products Corporation obtained a sample of the AQ Lot 5893 bioassay material from the NTP. This material was found to be mutagenic in the Ames test using the preincubation method of analysis (personal communication). When the bioassay material was purified, the mutagenic activity was lost. Mr. Cook informed the NTP of these results in writing.

Arkion Life Sciences using a different sample of the AQ Lot 5893 bioassay material supplied by the NTP undertook a second study. That study found that the AQ used in the NTP bioassay was contaminated with biologically significant amounts of mutagenic contaminants, including 9-NA and other organics (Butterworth *et al.*, 2001). These contaminants confirmed that the NTP AQ had been derived from coal tar. The NTP AQ test material exhibited substantial mutagenic activity in several Ames bacterial tester strains with and without metabolic activation using the plate incorporation methodology (Butterworth *et al.*, 2001). When the NTP-AQ was purified, however, the AQ no longer exhibited mutagenic activity (Butterworth et al., 2001).

Table 1. Mutagenicity and Purity Data for Anthraquinone

Testing Laboratory	Preparation	Percent Impurities	Conc.in strain TA98 (µg/plate)	Rev./plate *minus* S9
NTP	Coal tar derived AQ	3%	0	15
		(NTP TR 494	333	225
		1999)	1,000	723
			2,500	1,497
NTP	Coal tar derived AQ **Purified**	0%	0	12
		(NTP TR 494	1,000	13
		2004)	3,333	12
			10,000	12
Arkion	Coal tar derived AQ	0.65%	0	18
	Bioassay test material	(Butterworth et al.,	500	116
	Lot 5893	2001 & 2004)	1,000	213
			2,000	433
Arkion	Coal tar derived AQ	0%	0	14
	Bioassay test material	(Butterworth et al.,	500	11
	Lot 5893 **Purified**	2001 & 2004)	1,000	15
			2,000	22
NTP	Coal tar derived AQ	0.15%	0	7
	Bioassay test material	(NTP 12/9/04 meeting	1,000	4
	Lot 5893	presentation)	3,000	6
			10,000	6

Analytical Purity

The same AQ Lot 5893 bioassay material supplied by the NTP used in the mutagenicity studies was also analyzed by Arkion Life Sciences using a rigorous analytical procedure specifically designed to quantitative impurities in AQ-OX that can be missed by conventional techniques (Butterworth et al., 2004). Analysis showed that the level of contamination in the bioassay material was 0.65%. The individual component in the highest amount was 9-nitroanthracene (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%.

In contrast, a detailed analysis by the NTP of a different sample of the AQ Lot 5893 found a level of impurity of only 0.15% (presentation by Cynthia Smith at the NTP Technical Reports Subcommittee Review Meeting in Research Triangle Park, NC on December 9, 2004). The major contaminant was 0.1% 9-nitroanthracene. The reasons for the differing results are not known, but might be related to heterogeneity of the test material.

The 2004 Revised NTP TR 494

The mutagenicity issue had not been raised in the original NTP report, and the NTP peer-review committee was not aware of the contamination issue when they approved that report (NTP, 1999). In light of the new contamination concerns, however, the NTP withheld release of TR 494 and conducted their own purity and mutagenicity studies. A new TR 494 was drafted (NTP, 2004). In that report, the NTP confirmed that purified AQ was not a bacterial mutagen (Table 1).

Of particular concern was that quantitative mutagenicity and carcinogenicity potency estimates indicated that it was plausible that the contaminants alone in the NTP AQ bioassay could have been responsible for all of the observed carcinogenic activity (Butterworth et al., 2001; Butterworth et al., 2004). Despite this observation and the other published concerns, the NTP was inclined to dismiss the contamination issue. Thus, this important information was not presented in either the Title or the Abstract and could well have be overlooked by those not reading the entire Technical Report in detail (NTP, 2004).

The major argument used by the NTP for dismissing the contamination issue was that the primary contaminant, 9-NA, was a less potent bacterial mutagen in TA98 without S9 than the 2-hydroxyanthraquinone (2-OH-AQ) metabolite (NTP, 2004 - page 119). They concluded that one could ignore any contribution from the 9-NA since more mutagenic activity would come from the 2-OH-AQ metabolite. This argument fails on two counts. First, *purified* 2-OH-AQ is *not* mutagenic in TA98 minus S9 (Butterworth et al., 2004). The 2-OH-AQ used by the NTP was only about 90% pure and contaminants appear to have been responsible for the mutagenic activity (Table 2). The second reason that the argument fails is that an assay with 9-NA shows the chemical to be a potent mutagen in the human B-lymphoblastoid cell line that expresses P450 1A1 as well as the mouse lymphoma mutagenesis assay (Durant *et al.*, 1996; Butterworth *et al.*, 2004). This activity in mammalian cells overrides any results in bacteria and strengthens the argument that 9-NA may have significantly contributed to the tumor response.

Table 2. Mutagenicity and Purity Data for 2-Hydroxyanthraquinone

Testing Laboratory	Preparation	Percent Impurities	Conc. (μg/plate)	Rev./plate in strain TA98 *minus* S9
NTP	2-hydroxy-	10%	0	18

	anthraquinone	(NTP TR 494	100	246
		2004 and 12/9/04	200	160
		presentation)	333	169
Arkion	2-hydroxy-	0%	0	20
	anthraquinone	(Butterworth et al.,	100	12
		2004)	333	9
			1,000	7

The First Technical Report Subcommittee Review

It would be a disservice to the reader and user of the NTP report to imply in the title and abstract that pure AQ had been evaluated, when the degree to which contamination might have affected the study results was not known. Accordingly, on February 18, 2004 an NTP Technical Reports Review Subcommittee recommended the study title be changed from "Anthraquinone" to "Anthracene-Derived Anthraquinone" This was done to alert the reader that contamination issues should be considered in evaluating the study results.

The Second Technical Report Subcommittee Review

Following the February 28, 2004 review, the NTP conducted additional mutagenicity studies on the NTP coal tar derived AQ test material. They found that in their hands, their sample of Lot 5893 had no mutagenic activity in bacterial tester strains TA98, TA1537 or TA100 with or without metabolic activation using a preincubation protocol (presentation by Richard Irwin at the NTP Technical Reports Subcommittee Review Meeting in Research Triangle Park, NC on December 9, 2004) (Table 1).

Based on these new data, and the desire to avoid confusion for regulatory agencies, the NTP Technical Reports Subcommittee on December 9, 2004 reversed the title change for TR 494 from "Anthracene-Derived Anthraquinone" back to "Anthraquinone" and removed all references to any contamination issue in the study summary. The Subcommittee did, however, instruct the NTP to present a complete discussion of the contamination concerns in the body of the text.

The NTP Study Material May Not Have Been Homogeneous

A disturbing aspect of the above studies is that the samples of the coal tar derived AQ test material Lot 5893 provided by the NTP to Chemical Products Corporation and to Arkion Life Sciences both exhibited mutagenic activity in the Ames test and the magnitude of the responses were similar. In both cases, AQ purified from the test material was not mutagenic (Table 2). In contrast, the NTP studies of Lot 5893 showed no mutagenic activity (Table 2). All testing was done in duplicate by reputable commercial laboratories. The Chemical Products Corporation studies and the NTP studies were done using the preincubation protocol. The Arkion Life Sciences studies were done using the plate incorporation protocol. A similar discrepancy was found in the analytical studies. The NTP observed 0.15% contaminants for Lot 5893 (presentation by Cynthia Smith at the NTP Technical Reports Subcommittee Review Meeting in Research Triangle Park, NC on December 9, 2004). Detailed studies conducted by Arkion for Lot 5893 reported a level of 0.65% contaminants.

The most straightforward explanation of these apparently conflicting results is that the NTP test material was not homogeneous. Was Lot 5893 from one large container? Was the material evenly mixed? Was Lot 5893 supplied in many smaller containers? What is the analytical and mutagenic consistency from one container to the next? Determination of substantial heterogeneity in the test material could invalidate the bioassay. Given that small differences in the contamination level can result in dramatically different mutagenic activity (Table 1). It is imperative that the NTP address this issue.

References

Butterworth, B. E., Mathre, O. B., and Ballinger, K. (2001). The preparation of anthraquinone used in the National Toxicology Program cancer bioassay was contaminated with the mutagen 9-nitroanthracene. *Mutagenesis* 16, 169-177.

Butterworth, B. E., Mathre, O. B., Ballinger, K. E., and Adalsteinsson, O. (2004). Contamination is a frequent confounding factor in toxicology studies with anthraquinone and related compounds. *Internat. J. Toxicol.* 23, 355-344.

Durant, J. L., Busby, W. F., Lafleur, A. L., Pennman, B. W., and Crespi, C. L. (1996). Human cell mutagenicity of oxygenated, nitrated and unsubstituted polycyclic aromatic hydrocarbons associated with urban aerosols. *Mutat. Res.* 371, 123-157

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