Dr. Starr called the meeting to order at 2:21PM. NCSAB Members Drs. Thomas Starr, Woodhall Stopford, Wayne Spoo, and Man-Sung Yim were in attendance. Drs. Elaina Kenyon, James Gibson attended via teleconference.

Presentation: Mode of Action for Carcinogenicity of Acrylonitrile
Diana Wong, Ph.D., DABT
National Center for Environmental Assessment
Office of Research and Development
US Environmental Protection Agency

Dr. Wong, in her presentation:
- Reviewed the carcinogenicity of acrylonitrile
- Evaluated a direct mutagenic mode of action
- Evaluated an oxidative stress mode of action
- Evaluated other modes of action
- Drew conclusions on the mode of action for acrylonitrile

The Carcinogenicity of Acrylonitrile (AN)

In rodents, AN is a multi-site carcinogen, with target organs being:
- **Sprague-Dawley and F344 rats** being:
  - (inhalation) - brain, Zymbal gland, mammary gland, small intestine, tongue (Quast, JF; Schuetz, MF; Balmer, TS; et al. (1980b) A two-year toxicity and oncogenicity study with acrylonitrile following inhalation exposure of rats. Dow Chemical Co., Toxicology Research Laboratory, Midland, MI).


The Maltoni studies generated some discussion. It was stated that Maltoni seemed to be the only researcher finding liver tumors in adult animals. Maltoni found not only hepatomas but also angiosarcomas in both adult rats and those with early lifestage exposure. Hepatomas have only been found with exposure in utero and perinatal exposure. Early lifestage exposure was clarified (for the Maltoni study) as exposure of pregnant rats on gestation day 12 (offspring rats were exposed in utero). Two early lifestage exposure groups of rats were exposed postnatally: one up to 15 weeks, and the other up to 104 weeks. Hepatomas were found in both groups of rats.

- **B6C3F1 mice** (gavage study) being:

Human epidemiological studies have found that there was some association between occupational exposure and lung cancer deaths.
The metabolism of acrylonitrile was described as:

There are two main metabolic pathways for AN metabolism: (1) the oxidation by CYP2E1 of AN to form 2-cyanoethylene oxide, and (2) the conjugation of AN by glutathione (GSH), a detoxification pathway.

Dr. Wong stated that the key event in the AN mutagenic mode of action was the oxidation of AN by CYP2E1 to form an epoxide metabolite, 2-cyanoethylene oxide (CEO). This oxidative step occurs primarily in the liver, but it can occur at other sites as well (brain, forestomach, and intestinal mucosa). CEO interacts with DNA causing damage (including adduct formation, strand breaks, SCE, etc.) that can lead to tumor initiation.

Dr. Wong stated that the experimental evidence for a mutagenic mode of action is strong and consistent: there are studies that have found (1) DNA damage (chromosomal damage, DNA strand breaks, micronuclei) in occupationally exposed workers; (2) DNA damage in the rat (bone marrow, stomach, brain, liver, testes, forestomach, colon, kidney, bladder, and lung) as well as the ddY mouse (forestomach, bladder and brain); and (3) positive in vitro mutation assay results using bacteria, fungi, insects, as well as animal and human cell cultures.

In addition, Dr. Wong pointed out that (1) the mutagenicity/genotoxicity of AN appears to be specific and occurs in the absence of overt toxicity; (2) the temporal relationship with carcinogenicity has not been adequately explored, but that mutagenicity occurs in short-term assays (before tumor formation); (3) there is a dose-response relationship for in vivo AN exposure and DNA damage; and (4) a mutagenic mode of action appears biologically plausible and coherent (AN is metabolized to a reactive epoxide, CEO, which can ultimately cause mutation and cancer).
Experimental evidence exists for the formation of DNA adducts with exposure to AN. After a 40-day incubation, AN interacted with calf thymus DNA in vitro at concentrations >1M, forming mainly 1-carboxyethyl adenosine, 7-cyanoethyl-guanine, imidazole ring-opened 7,9-bis cyanoethyl guanine, 3-cyanoethyl thymine, and N⁶-cyanoethyl adenine (Solomon et al., 1984). The oxidative metabolite, CEO (1.36M) formed DNA adducts in 3 hours after incubation with calf thymus DNA. These adducts included: N⁷-(oxoethyl)guanine, N³-(2-hydroxyl-2-carboxylethyl)deoxyuridine (3-HOCE-dUrd) and small amounts of adenine and thymine adducts (Solomon, JJ; Singh, US; and Segal, A. (1993) In vitro reactions of 2-cyanoethylene oxide with calf thymus DNA. Chem-Biol Interact 88: 115-135).

AN is able to induce mutations in bacterial strains of salmonella typhimurium through base substitution and frameshift mutation. S9 enzymes are required for this response. Mutagenicity is also demonstrated in tryptophan-dependent E. coli WP2 series. In strains deficient in DNA repair, positive enhancement of response has been observed.

AN exposure also induced mutations at the Tk+/− locus in mouse lymphoma cell assays and in human lymphoblastoid TK6 cells (with S9). CEO is 10 times more potent than AN in this mutagenic process.

At the HPRT locus, AN has been found to be mutagenic in TK6 cells, with the majority of mutations exhibiting specific loss of exons from the coding region.

AN cytogenicity in cultured mammalian cells has been demonstrated by (1) sister chromatid exchanges (SCE) in human lymphocytes (with S9), in human bronchial epithelium cells (without S9), and in Chinese hamster ovary (CHO) cells (with S9), or co-cultured with rat hepatocytes; (2) chromosomal aberrations in Chinese hamster ovary (CHO) cells (with or without S9) and liver fibroblasts, and in Chinese hamster lung fibroblasts (without S9); and (3) in micronuclei: CHO cells (without S9).

Dr. Wong explained the evidence for DNA binding and subsequent damage in rat studies. Using Sprague-Dawley rats, it has been reported that there is DNA and RNA binding in the brain, stomach, and liver after a single oral dose. DNA alkylation is higher in the brain or in the stomach than the liver (119 or 81 vs 25 pmol/mg) (Farooqui, MY; Ahmed, AE. (1983a) In vivo interactions of acrylonitrile with macromolecules in rats. Chem Biol Interact 47:363-371); that there is DNA binding in the testes, lung, and gastric tissue after a single oral dose of 46.5 mg/kg (Ahmed et al. 1992a; Ahmed et al. 1992b); that there is no DNA binding in the liver and brain detected after a single intraperitoneal dose of 0.6 mg/kg [2,3-14C]CEO was administered to one F344 rat (Hogy, LL; Guengerich, FP. (1986) In vivo interaction of acrylonitrile and 2-cyanoethylene oxide with DNA in rats. Cancer Res 46:3932-3938). (N⁷-(2-oxoethyl)guanine adduct was detected in liver DNA when treated with 6 mg/kg CEO or 50 mg/kg AN intraperitoneally); and that there are micronuclei in bone marrow after 98 or 124 mg/kg AN i.v., but not after oral doses up to 40 mg/kg (Wakata, A; Miyamae, Y; Sato, S-I; et al. (1998) Evaluation of the rat micronucleus test with bone marrow and peripheral blood: summary of the 9th collaborative study by CSGMT/JEMS.MMS. Environ Mol Mutagen 32:84-100). Using Wistar rats, it has been found that there is DNA damage (by comet assay) in the forestomach, colon, kidney, bladder and lung (Sekihashi, K; Yamamoto, A; Matsumura, Y; et al. (2002) Comparative investigation of multiple organs of mice and rats in the comet assay. Mutat Res 517:53-75). Using F344 rats, it is reported that a dominant lethal assay (for germ cell mutation) was negative after 60 mg/kg-day AN for 5 days (Working, PK; Bentley, KS; Hurtt, ME; et al. (1987) Comparison of the dominant lethal effects of acrylonitrile and acrylamide in male F344 rats. Mutagenesis 2:215-220).

There are several limitations of these DNA adduct studies: (1) most major adducts from CEO incubation studies have not been measured. These studies were largely done in the 1980s and 1990s, and researchers did not know exactly what to look for; (2) high detection limits; more sensitive methods were not used; and (3) No DNA adduct studies were conducted with repeated dosing in rats.

Insofar as DNA repair in rats is concerned, Dr. Wong stated that unscheduled DNA synthesis (UDS) after oral dosing is reported in rat lung (Ahmed, AE; Abdel-Aziz, AH; Abdel-Rahman, SZ; et al. (1992a) Pulmonary toxicity of acrylonitrile: covalent interaction and effect on replicative and unscheduled DNA synthesis in the lung. Toxicology 76:1-14), in gastric tissue (Abdel-Rahman, SZ; Nouraldeen, AM; Abo-Elwafa, AA; et al. (1994a) Acrylonitrile-induced reversible inhibition
of uridine uptake by isolated rat intestinal epithelial cells. Toxicol Vitro 8:139-143), and in the
testis (Ahmed, AE; Abdel-Rahman, SZ; Nouraldeen, AM. (1992b) Acrylonitrile interaction with testicular
DNA in rats. J Biochem Toxicol 7:5-11). Further that there is UDS in the liver, but not in the brain after
50 mg/kg AN or 6 mg/kg CEO was administered i.p. (Hogy, LL; Guengerich, FP. (1986) In vivo
interaction of acrylonitrile and 2-cyanoethylene oxide with DNA in rats. Cancer Res 46:3932-
3938), and further, that UDS was not detected by autoradiography in primary cultures of hepatocytes
or spermatocytes from F344 rats after single oral doses of 75 mg/kg or 5 daily doses of 60 mg/kg-day
AN (Butterworth, BE; Eldridge, SR; Sprankle, CS; et al. (1992) Tissue-specific genotoxic

In mouse studies, DNA damage was reported in the forestomach, colon, bladder, lung and brain of
male ddY mice (20 mg/kg, i.p. (Sekihashi, K; Yamamoto, A; Matsumura, Y; et al. (2002)
Comparative investigation of multiple organs of mice and rats in the comet assay. Mutat Res
517:53-75)). SCE was reported in bone marrow cells of Swiss mice (after administration of 7.5 or 10
mg/kg AN i.p. (Fahmy, MA. (1999) Evaluation of the genotoxicity of acrylonitrile in different
tissues of male mice. Cytologia 64:1-9)). There was only a small increase in micronuclei in the
bone marrow of mice after administration of 5.6-45 mg/kg AN i.p. (Morita, T; Asano, N; Awogi, T; et
al. (1997) Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens
(groups 1,2A and 2B): The summary report of the 6th collaborative study by CSGMT/JEMS
MMS. Collaborative study of the micronucleus group test. Mammalian Mutagenicity Study
Group. Mutat Res 389:3-122), but no increase was detected after i.v. administration of AN. A single
oral dose of 7.75 mg/kg AN or a repeated dose of 7.75 mg/kg for 3 or 5 days induced chromosomal
aberrations in mouse spermatocytes, bone-marrow and spleen cells (Fahmy, MA. (1999) Evaluation
of the genotoxicity of acrylonitrile in different tissues of male mice. Cytologia 64:1-9). Earlier
studies had shown, however, that there were no chromosomal aberrations in bone marrow cells of
mice after dosing with 20 mg/kg-day AN for up to 21 days or i.p. (Rabello-Gay, MN; Ahmed, AE.
Sharief, Y; Brown, AM; Backer, LC; et al. (1986) Sister chromatid exchange and chromosome
aberration analyses in mice after in vivo exposure to acrylonitrile, styrene, or butadiene
monoxide. Environ Mutagen 8:439-448; Leonard, A; Garny, V; Poncelet, F; et al. (1981)
was reported in male mice (30 mg/kg AN (Leonard, A; Garny, V; Poncelet, F; et al. (1981)
Mutagenicity of acrylonitrile in mouse. Toxicol Lett 7:329-334)).

It was reported that DNA damage in occupationally exposed humans was evidenced by (1) increased
micronuclei in buccal mucosal cells (average AN of 0.522 mg/m3 and 1.998 mg/m3 for 1-33 years,
and in blood lymphocytes (average AN of 1.998 mg/m3 for 1-31 years (Fan W, Wang WL, Ding S,
mucosal cells in assessing the genetic damage of workers exposed to Acrylonitrile. Zhonghua Lao
Dong Wei Sheng Zhi Ye Bing Za Zhi. 2006 Feb;24(2):106-8)); (2) increased chromosomal
aberrations were found in production and maintenance workers in an AN fiber production plant
(Borba, H; Monteiro, M; Proenca, MJ; et al. (1996) Evaluation of some biomonitoring markers
in occupationally exposed populations to acrylonitrile. Teratog Carcinog Mutagen 16:205-218),
and in sperm DNA strand breakage and sex chromosome aneuploidy (0.8 mg/m3 AN for 2.8 years
(Xu, DX; Zhu, QX; Zheng, LK; et al. (2003) Exposure to acrylonitrile induced DNA strand
breakage and sex chromosome aneuploidy in human spermatozoa. Mutat Res 537:93-100)). An
increase in the relative number of insertions was found in chromosome of cultured lymphocytes (O
Beskid, Z Dušek, I Solanský, R J Srám. The effects of exposure to different clastogens on the
pattern of chromosomal aberrations detected by FISH whole chromosome painting in
occupationally exposed individuals. Mutat Res. 2006 Feb 22;594(1-2):20-9). It was pointed out
that these epidemiological studies did not discuss any potential co-exposures of workers that could
occur. There is little doubt that in an occupational setting, there are exposures to multiple chemicals.
To strengthen the case for DNA damage resulting from AN exposure, effects from all other exposures
must be categorized, assessed and evaluated.

Dr. Wong reported that single AN doses that cause DNA damage, chromosome aberrations,
mutations and UDS in rodents are in concordance with tumorigenic doses in chronic bioassays. To
support this, it was explained that (1) doses causing chromosome aberrations in spermatocytes of
Swiss mice (15.5 or 31 mg/kg AN or 5 daily doses of 7.75 mg/kg AN) or in spleen cells and bone
marrow cells (7.75 mg/kg) are comparable to the tumorigenic doses in B6C3F1 mice (2.5 to 20 mg/kg-day in a 2 year bioassay); and (2) although by a different route, doses causing DNA damage in rats (30 mg/kg i.p.), and in male ddY mice (20 mg/kg i.p.) are comparable to tumorigenic oral doses in rats and mice.

Additional evidence supportive of a mutagenic mode of action comes from the determination that the pattern of tumors observed is consistent with DNA-reactive chemicals. The tumors observed are systemic and occur at multiple sites. For example, Zymbal gland tumors in rats commonly occur with carcinogens that are also mutagens (23 of 27 chemicals that induce tumors in this gland are mutagenic in the Ames assay, while all 27 are multi-site carcinogens). Tumors sometimes occur at nontoxic doses or concentrations, and sometimes occur after less-than-lifetime exposure. In an inhalation study, rats were exposed for only 52 weeks, found gliomas in 20- or 40-ppm exposed groups at week 84 and week 63.5, forestomach tumors (latency 104-124 weeks), and Zymbal gland carcinomas (latency 77-102 weeks) (Maltoni, C; Ciliberti, A; Di, MV. (1977) Carcinogenicity bioassays on rats of acrylonitrile administered by inhalation and by ingestion. Med Lav 68:401-411). Generally, the ratio of benign to malignant tumors is small, and tumors occurred with higher frequencies in rats with early-life exposure.

The key events for an oxidative mode of action were then discussed. Reactive oxygen species (ROS) are generated either directly from the oxidant, indirectly during metabolism, or via activation of endogenous sources, and can interact with and damage DNA leading to gene mutation for tumor initiation. Lipids and proteins can also be also damaged by ROS. ROS can also function as tumor promoters, via modification of gene expression, through modulation of signaling pathways and transcription factors [e.g. NFκB, AP-1]] or changes in DNA methylation status. In vitro evidence for such a mode of action includes: (1) dose-dependent increase in transformation of SHE cells by AN associated with increased 8-hydroxy-2'-deoxyguanosine (8-OHdG) in DNA (Zhang et al., 2000); (2) α-tocopherol blocked both 8-OHdG formation and cell transformation; (3) 8-OHdG levels increased in DNA of AN-exposed cultured rat astrocytes (Kamendulis, LM; Jiang, J; Zhang, H; et al. (1999) The effect of acrylonitrile on gap junctional intercellular communication in rat astrocytes. Cell Biol Toxicol 15:173-183), and human astrocytes (Jacob, S; Ahmed, AE. (2003) Acrylonitrile-induced neurotoxicity in normal human astrocytes: oxidative stress and 8-hydroxy-2'-deoxyguanosine formation. Toxicol Mech Methods 13:169-179) but not in cultured rat hepatocytes (Kamendulis, LM; Jiang, J; Zhang, H; et al. (1999) The effect of acrylonitrile on gap junctional intercellular communication in rat astrocytes. Cell Biol Toxicol 15:173-183); (4) increase in hydroxyl radical formation, and decrease in levels of glutathione and antioxidant enzymes in exposed glial cells (Kamendulis, LM; Jiang, J; Xu, Y; et al. (1999a) Induction of oxidative stress and oxidative damage in rat glial cells by acrylonitrile. Carcinogenesis 20:1555-1560) and human astrocytes (Jacob, S; Ahmed, AE. (2003) Acrylonitrile-induced neurotoxicity in normal human astrocytes: oxidative stress and 8-hydroxy-2'-deoxyguanosine formation. Toxicol Mech Methods 13:169-179).

In the rat brain, as evidenced by DNA 8-OHdG levels, there was a dose-related increase in the cortex of male Sprague-Dawley rats exposed to ≥30 ppm AN in drinking water for 14-90 days (Jiang, J; Xu, Y; Klaunig, J E. (1998) Induction of oxidative stress in rat brain by acrylonitrile (ACN). Toxicol Sci 46:333-341), and a two-fold increase in Sprague-Dawley rats (30 or 300 ppm AN for 21 days) but smaller, statistically insignificant increases in F344 rats exposed to ≤100 ppm AN in drinking water for the same duration (21 days) (Whysner, J; Ross, PM; Conaway, CC; et al. (1998) Evaluation of possible genotoxic mechanisms for acrylonitrile tumorigenicity. Regul Toxicol Pharmacol 27:217-239). However, brain DNA 8-OHdG levels do not seem to be associated with tumor incidence in male F344 rats (Table 1), or Sprague-Dawley rats (Table 2).
Table 1

**Brain DNA 8-OHdG Not Associated with Tumor Incidence in Male F344 Rats**

<table>
<thead>
<tr>
<th>Dose Group (ppm)</th>
<th>8-OHdG (mol/10^5 mol dG)</th>
<th>Incidence of Brain Astrocytoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.79 ± 0.37</td>
<td>2/160</td>
</tr>
<tr>
<td>1</td>
<td>0.84 ± 0.37</td>
<td>2/80</td>
</tr>
<tr>
<td>3</td>
<td>1.07 ± 0.37</td>
<td>1/78</td>
</tr>
<tr>
<td>10</td>
<td>1.04 ± 0.37</td>
<td>2/80</td>
</tr>
<tr>
<td>30</td>
<td>1.03 ± 0.37</td>
<td>10/79</td>
</tr>
<tr>
<td>100</td>
<td>1.06 ± 0.37</td>
<td>21/76</td>
</tr>
</tbody>
</table>

Table 2

**Brain DNA 8-OHdG Not Associated with Tumor Incidence in Male Sprague-Dawley Rats**

<table>
<thead>
<tr>
<th>Dose Group (ppm)</th>
<th>8-OHdG (mol/10^5 mol dG)</th>
<th>Incidence of Brain Astrocytoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.62 ± 0.08</td>
<td>1/80</td>
</tr>
<tr>
<td>3</td>
<td>0.86 ± 0.41</td>
<td>No data</td>
</tr>
<tr>
<td>30</td>
<td>1.35 ± 0.49</td>
<td>No data</td>
</tr>
<tr>
<td>35</td>
<td>No data</td>
<td>8/47</td>
</tr>
<tr>
<td>100</td>
<td>No data</td>
<td>19/48</td>
</tr>
<tr>
<td>300</td>
<td>1.29 ± 0.10</td>
<td>23/48</td>
</tr>
</tbody>
</table>

Additionally, there is no consistent disruption of antioxidant defense. There are persistent decreases in catalase in cortex for 50 -200 ppm AN groups, and small decrease in GSH levels and SOD activities in all dose groups after 14 days of AN (Jiang, J; Xu, Y; Klaunig, J E. (1998) Induction of oxidative stress in rat brain by acrylonitrile (ACN). Toxicol Sci 46:333-341). There were no changes in brain GSH, catalase and glutathione peroxidase activities in Sprague-Dawley rats after 21 days of 3 - 300 ppm AN. Cysteine level was increased in the 300 ppm group, and there was no change in brain GSH and cysteine levels in F344 rats (1- 300 ppm AN for 21 days (Whysner, J; Ross, PM; Conaway, CC; et al. (1998b) Evaluation of possible genotoxic mechanisms for acrylonitrile tumorigenicity. Regul Toxicol Pharmacol 27:217-239)). No lipid peroxidation was found in the rat brain.

Is there a role for cyanide? No changes were detected in cytochrome oxidase activity in brain mitochondria isolated from treated Sprague-Dawley rats and F344 rats (Whysner, J; Steward, RE, III; Chen, D; et al. (1998a) Formation of 8-oxodeoxyguanosine in brain DNA of rats exposed to acrylonitrile. Arch Toxicol 72:429-438), and cyanide-induced metabolic hypoxia was probably not involved in the ROS generation by AN.

The evidence does not show specificity and consistency in increased oxidative DNA damage and AN-induced tumor formation.

Another candidate mode of action is inhibition of intercellular communication. AN (0.1 to 1.0 mmol/L) inhibited gap junction intercellular communications (GJIC) in D1 TNC1 astrocytes (a rat astrocyte transformed cell line). The inhibition was reversible and reduced by co-treatment with vitamin E or oxothiazolidine-4-carboxylic acid (OTC), a precursor for the synthesis of glutathione, in the culture medium. GJIC was not inhibited in cultured rat hepatocytes (Kamendulis, LM; Jiang, J; Xu, Y; et al. (1999a) Induction of oxidative stress and oxidative damage in rat glial cells by acrylonitrile. Carcinogenesis 20:1555-1560). There are no in vivo studies available. This mode of action has not been adequately studied.
Cell proliferation was also reviewed by Dr. Wong. Hyperplasia of the squamous epithelium of the forestomach was observed in chronic oral studies of rats at and above the lowest dose of 0.09 mg/kg-day (Johannsen, FR; Levinskas, GJ. (2002b) Chronic toxicity and oncogenic dose-response effects of lifetime oral acrylonitrile exposure to F344 rats. Toxicol Lett 132:221-247; Quast, JF. (2002) Two-year toxicity and oncogenicity study with acrylonitrile incorporated in the drinking water of rats. Toxicol Lett 132:153-196); and in mice at and above 10 mg/kg-day (NTP, 2001). AN induced a dose-dependent increase in epithelial proliferation in the forestomach of F344 rats treated with 0, 11.67 or 22.8 mg/kg-day AN for 6 weeks (Ghanayem, BI; Elwell, MR; Eldridge, SR. (1997) Effects of the carcinogen, acrylonitrile, on forestomach cell proliferation and apoptosis in the rat: Comparison with methacrylonitrile. Carcinogenesis 18:675-680), although no hyperplasia was observed in the glandular stomach or the liver of treated rats. An associated increase (60%) in hyperplasia of the forestomach squamous mucosa was observed in the high dose group, and an increase in forestomach apoptosis was observed at the high dose group. No hyperplasia observed in the brain of treated rats in chronic studies. In in vitro studies with rat astrocytes, indices of cytotoxicity after 24 hours of AN exposure occurred at higher concentrations (2.5, 5.0, and 10.0 mM) than increased 8-OHdG levels in DNA (0.01, 0.1, and 1.0 mM) (Kamendulis, LM; Jiang, J; Xu, Y; et al. (1999a) Induction of oxidative stress and oxidative damage in rat glial cells by acrylonitrile. Carcinogenesis 20:1555-1560). Available data do not support a prominent role for cytotoxic or mitogenic modes of action in AN-induced rat brain tumors.

The mode of action for tumor formation in the forestomach was reviewed. DNA damage was reported in the forestomach after single i.p. dose in rats (30 mg/kg) and mice (20 mg/kg) (Sekihashi, K; Yamamoto, A; Matsumura, Y; et al. (2002) Comparative investigation of multiple organs of mice and rats in the comet assay. Mutat Res 517:53-75). However, no significant oxidative DNA damage was found in the forestomach of rats exposed up to 300 ppm AN in drinking water for 21 days (Whysner, J; Ross, PM; Conaway, CC; et al. (1998b) Evaluation of possible genotoxic mechanisms for acrylonitrile tumorigenicity. Regul Toxicol Pharmacol 27:217-239). It was concluded that cell proliferation might contribute in addition to direct mutagenic mode of action for forestomach tumors.

Dr. Wong concluded by summarizing the evidence for a carcinogenic mode of action for AN:

(1) Data gaps still exist in our current understanding of the mode of action for carcinogenicity of AN.
(2) There is sufficient experimental evidence to support direct mutagenicity as the key mode of action.
(3) Other modes of action may contribute, but limited data do not appear supportive at this time.
(4) Indirect mutagenicity via oxidative DNA damage is plausible, but experimental evidence indicates that it is not likely to be an important mode of action.
(5) There are no published studies that investigate the potential tumor promoting effect of AN via alteration of gene expression.

Dr. Wong said that the translated Chinese AN studies could be supplied to the NCSAB. See attached.

Dale Strother, representing the AN Group, said that a meeting was being organized to review the carcinogenicity of AN in Washington, DC, May 20-22, 2007.

Other Business

The minutes of the 121st meeting of the NCSAB were approved as amended.

The NCSAB requested that the offer by Linda Birnbaum of EPA to review their PFOA research for the NCSAB be accepted and that a meeting be set up.
Agenda for the Next NCSAB Meeting

The next meeting of the NCSAB will be held at the 2728 Capital Building (known formerly as Parker-Lincoln) in Room 1A224 (Environmental Health Training Room) at 2:00PM on THURSDAY, May 10, 2007. Dr. Chris Lau of EPA/NHEERL is scheduled to speak on EPA’s research on Perfluorooctanoic Acid.

The meeting was adjourned at 4:20PM.

Respectfully submitted,

Reginald C. Jordan, Ph.D., CIH
Liaison, Science Advisory Board

**Abstract**

Objective To investigate the application of micronucleus test of buccal mucosal cells in monitoring the genetic effect of acrylonitrile in the population exposed to the acrylonitrile. Methods Forty-one healthy male workers in a chemical factory in Shanghai were selected as the low concentration acrylonitrile exposed group while forty-seven healthy male workers in an acrylonitrile factory in Shanghai were selected as the intermediate concentration acrylonitrile exposed group. At the same time, thirty-four male workers who had no toxicant exposure and lived in the same community were selected as the control group. The micronucleus test in buccal mucosal cells and lymphocytes were used respectively for assessing the genetic damage status of these men. Results The rate of micronucleus in buccal mucosal cells in both acrylonitrile groups (the low concentration group: 3.68 % ± 2.72 %, the intermediate concentration group: 4.00 % ± 2.38 %) was significantly higher than that in the control group (2.05 % ± 2.20 %) (P < 0.05). The rate of micronucleus in the intermediate concentration group (4.23 % ± 3.34 %) was significantly higher than that in the control group (2.48 % ± 1.46 %) (P < 0.05). There was the correlation between the micronucleus test of buccal mucosal cells and the micronucleus test of the lymphocytes in the peripheral blood in the acrylonitrile exposed population (r = 0.299 ~ 0.539, P < 0.05). Conclusion The micronucleus test of buccal mucosal cells replacing the micronucleus test of the lymphocytes in the peripheral blood can be used as one of the screening indexes in the surveillance of the genetic damage in the acrylonitrile exposed population.

**Key words** Acrylonitrile ; Mouth mucosa ; Monocyte ; Micronucleus test

Acrylonitrile (ACN) is an important monomer in organic syntheses and is widely used as a raw material in the production of nitrile fibers, butadiene-acrylonitrile rubbers, ABS engineering plastics, and certain synthetic resins. ACN belongs to a group of highly toxic substances. It is a colorless, highly flammable, very volatile liquid. Accordingly, accidental acute poisoning can easily occur during its production and/or use. The results of previous studies indicate that ACN has definite mutagenicity and is considered to be a latent carcinogen [1, 2]. Therefore, among those with long-term exposure to ACN, it is important to discover any genetic damage at an early stage. We used the micronucleus test as the index for genetic damage among people exposed to ACN in order to carry out a preliminary investigation of the genetic effects of ACN among those with occupational contact with ACN using buccal mucosal cells as the study material.

**STUDY SUBJECTS AND METHODS**

1. **Study Subjects**: 41 male subjects were selected randomly from among the workers in a certain chemical engineering plant in Shanghai who had had direct contact with ACN. Their average age was 37.4 ± 7.3 years and the contact time was in the range of 1-33 years with an average time of 15.7 ± 8.7 years. The rate of alcohol use was 41.5% (17/41) and the rate of cigarette smoking was 65.9% (27/41). 47 male workers having direct contact with ACN were also selected from a certain nitrile fiber plant. Their average age was 39.8 ± 8.5
years and the contact time was in the range of 1-33 years with an average time of 17.2 ± 9.3 years. The rate of alcohol use was 66.0% (31/47) and the rate of cigarette smoking was 63.8% (30/47). Based on the ACN concentration measured in the workplace air, the workers from the chemical engineering plant were classified as a low concentration contact group (average concentration 0.522 mg/m$^3$) and the workers from the nitrile fiber plant were classified as a medium concentration contact group (average concentration 1.998 mg/m$^3$). 31 healthy male workers who had no contact with any known mutagenic toxin or ACN were also selected from the same district and used as the control group. Their average age was 37.2 ± 8.6 years and the average number of years of working was 16.7 ± 8.8 years. The rate of alcohol use was 71.0% (22/31) and the rate of cigarette smoking was 61.3% (19/31).

2. Monitoring ACN Concentration in the Workplace Air: The ACN concentration in the air of the two workplaces was measured by the safety and environmental protection unit of the respective plant based on the "Workplace Air -- Determination of Acrylonitrile -- Thermal Desorption Gas Chromatographic Method" (GB/T [Chinese Industrial Standard -- Tr. Ed.] 16099-1995) [3] at the specified point and at the specified time. The determination method used was thermal desorption gas chromatography and the period of monitoring was 1997 to 1999. Analysis of the Workplace Environmental Characteristics of the Two Plants: In the chemical engineering plant, ACN is produced by the oxidation of propylene, ammonia, and air -- ACN was the final product. The entire propylene-ammonia oxidation process was carried out in a closed system of pipes and automated technology. The environment was a frame-type unrestricted work environment. The chance of coming into contact with ACN was primarily at the time of periodic on-site sampling and pipe inspection, even though the ACN concentration level was relatively low. In the nitrile fiber plant, ACN was used as a raw material in the synthesis of polyacrylonitrile by polymerization and various nitrile fibers were processed. During the production processes, ACN monomer escaped in large quantities. Because the processes were carried out indoors, the ACN concentration in the workplace air was relatively high. Table 1 shows the results of monitoring the ACN concentration in the workplace air.
TABLE 1. RESULTS OF MONITORING OF THE ACRYLONITRILE CONCENTRATION IN THE WORKPLACE AIR

<table>
<thead>
<tr>
<th>Plant</th>
<th>Number of Samples</th>
<th>Range</th>
<th>Mathematical Average ($x$)</th>
<th>Geometric Average ($Gx$)</th>
<th>Median ($M$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Engineering Plant</td>
<td>390</td>
<td>0.000-3.700</td>
<td>0.522</td>
<td>0.255</td>
<td>0.353</td>
</tr>
<tr>
<td>Nitrile Fiber Plant</td>
<td>570</td>
<td>0.000-18.100</td>
<td>1.998</td>
<td>1.986</td>
<td>1.853</td>
</tr>
</tbody>
</table>

3. **Rate of Occurrence of Micronuclei in Buccal Mucosal Cells (OMN).** The test subjects were asked to rinse their mouth with clean water and to use a sterilized wooden tongue depressor to scrape the mucous membrane on the inside of both cheeks. The second scrapings were washed into a 5-mL centrifuge tube with the use of 2 mL of a buffer solution (0.01 mole/L Tris·HCl, 0.1 mole/L EDTA, 0.02 mole/L NaCl). After gentle tapping for homogeneous mixing, the tube was centrifuged at 1,000 rpm for 10 minutes. The supernatant fluid was discarded. These processes were repeated once and the cells were then diluted to $1 \times 10^6$ to $2 \times 10^6$ cells/L. About 100 µL of this cell suspension was dropped on a glass slide, air-dried, fixed with anhydrous methanol for 15 minutes, and stained with Y. Ding [phonetic spelling; unverified -- Tr. Ed.] fluorescent orange dye. The sample was examined under a high magnification microscope. 1,000 buccal mucosal epithelial cells with intact cell membrane and nuclear membrane were read for each sample and the rate of occurrence of micronuclei was recorded.

4. **Rate of Occurrence of Micronuclei in Peripheral Blood Lymphocytes (BMN).** A 0.5 mL blood sample was collected and added to an equal volume of 0.5% by weight of methylcellulose. After homogeneous mixing, the mixture was placed in a 37°C water bath for spontaneous sedimentation for 30 minutes and then centrifuged. The procedures used for making and reading the slides were the same as those used for the OMN determinations.

5. **Statistical Methods.** Because data regarding the rate of occurrence of OMN and BMN did not follow a normal distribution, all data were subjected to square root conversion before being used in the statistical analysis. A multivariate linear regression was used in the multiple factor analysis. SPSS 10.0 software was used for all the statistical processes.
RESULTS

1. **Comparison of the Rate of Occurrence of OMN and BMN in Groups with Different Contact Doses.** The rate of occurrence of OMN in the low and medium concentration contact groups was compared with that in the control group; the rates in both contact groups were found to be statistically different from the rate in the control group \((P < 0.05)\). The rate of occurrence of BMN in the medium concentration contact group was found to be statistically different from that in the control group \((P < 0.05)\). In the low concentration contact group, the rate of occurrence of OMN was clearly higher than the rate of occurrence of BMN and the difference was statistically significant \((P < 0.05)\). See Table 2.

**TABLE 2. RATE OF OCCURRENCE OF MICRONUCLEI IN 2 KINDS OF CELLS IN POPULATIONS HAVING CONTACT WITH ACRYLONITRILE (‰, \(\bar{X} \pm s\)).**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Cases</th>
<th>Rate of Occurrence of Micronuclei (‰)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>buccal mucosal cells</td>
<td>peripheral blood lymphocytes</td>
</tr>
<tr>
<td>control group</td>
<td>31</td>
<td>2.03 ± 2.20</td>
<td>2.48 ± 1.46</td>
</tr>
<tr>
<td>low concentration contact group</td>
<td>41</td>
<td>3.68 ± 2.72*</td>
<td>2.44 ± 2.06</td>
</tr>
<tr>
<td>medium concentration contact group</td>
<td>47</td>
<td>4.00 ± 2.38*</td>
<td>4.23 ± 3.34*</td>
</tr>
</tbody>
</table>

Comparison with the Control Group: *\(P < 0.05\); Comparison with the Rate of Occurrence of Micronuclei in Peripheral Blood Lymphocytes in the same group, \#\(P < 0.05\).

2. **Results of Multivariate Linear Regression Analysis.** In order to investigate the relationship between ACN exposure and the rate of occurrence of micronuclei and to eliminate the possibility of the presence of contaminating factors, a progressive linear regression analysis was carried out. It was hoped that an overall conclusion could be made based on the results of this analysis. The variables analyzed and values obtained are listed in Table 3. The results clearly indicate that the cumulative contact amount of ACN, the recent contact amount of ACN, and the amount of cigarette smoking are important influencing factors on the rate of occurrence of OMN and BMN. See Tables 4 and 5.
TABLE 3. SELECTION OF VARIABLES USED IN MULTIVARIATE LINEAR REGRESSION ANALYSIS AND VALUES OBTAINED AND THEIR UNITS.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>Value Obtained and Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>rate of occurrence of micronucleus in buccal mucosal cells (‰)</td>
<td>cumulative contact amount of ACN (x₁)</td>
<td>mg</td>
</tr>
<tr>
<td>rate of occurrence of micronucleus in peripheral blood lymphocytes (‰)</td>
<td>recent contact amount of ACN (x₂)</td>
<td>mg</td>
</tr>
<tr>
<td>use of alcohol (x₃)</td>
<td>0 = no use; 1 = use</td>
<td></td>
</tr>
<tr>
<td>amount of cigarette smoking (x₄)</td>
<td>cigarettes</td>
<td></td>
</tr>
<tr>
<td>age (x₅)</td>
<td>years</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4. RESULTS OF A MULTIVARIATE LINEAR REGRESSION ANALYSIS OF THE RATE OF OCCURRENCE OF MICRONUCLEI IN BUCCAL MUCOSAL CELLS.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Partial Regression Coefficient</th>
<th>Regression Coefficient Standard Deviation</th>
<th>P Value</th>
<th>Determining Coefficient Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>constants</td>
<td>1.255</td>
<td>0.450</td>
<td>&lt; 0.05</td>
<td>0.399</td>
</tr>
<tr>
<td>cumulative contact amount of ACN (x₁)</td>
<td>6.057 × 10⁻⁶</td>
<td>0.000</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>amount of cigarette smoking (x₄)</td>
<td>2.133 × 10⁻⁶</td>
<td>0.000</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>use of alcohol (x₃)</td>
<td>−0.183</td>
<td>0.163</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>age (x₅)</td>
<td>6.678 × 10⁻⁶</td>
<td>0.012</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>constants</td>
<td>0.892</td>
<td>0.424</td>
<td>&lt; 0.05</td>
<td>0.380</td>
</tr>
<tr>
<td>recent contact amount of ACN (x₂)</td>
<td>2.006 × 10⁻¹</td>
<td>0.001</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>amount of cigarette smoking (x₄)</td>
<td>2.153 × 10⁻⁶</td>
<td>0.000</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>use of alcohol (x₃)</td>
<td>−0.174</td>
<td>0.164</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>age (x₅)</td>
<td>1.607 × 10⁻²</td>
<td>0.011</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Cumulative contact amount of ACN = yearly dose of ACN at the workplace × years of working × effective amount of air passage to the lung + average value of ACN in tobacco × number of cigarettes smoked per year × years of cigarette smoking; recent contact amount of ACN = yearly dose of ACN at the workplace × life of buccal mucosal cells × effective amount of air passage to the lung + average value of ACN in tobacco × number of cigarettes smoked per year × life of buccal mucosal cells.
Cumulative contact amount of ACN = yearly dose of ACN at the workplace × years of working × effective amount of air passage to the lung + average value of ACN in tobacco × number of cigarette smoked per year × years of cigarette smoking; recent contact amount of ACN = yearly dose of ACN at the workplace × life of buccal mucosal cells × effective amount of air passage to the lung + average value of ACN in tobacco × number of cigarette smoked per year × life of buccal mucosal cells

3. Analysis Results of the Relationship Between the Rate of Occurrence of OMN and BMN. The results shown in Table 6 indicate that the relationship between the rate of occurrence of OMN and BMN was statistically significant ($r = 0.299$ to $0.359$, $P < 0.05$).

TABLE 6. ANALYSIS OF THE RELATIONSHIP BETWEEN THE RATE OF OCCURRENCE OF OMN AND BMN IN POPULATIONS HAVING CONTACT WITH ACRYLONITRILE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Relationship Between the Rate of Occurrence of OMN and BMN</th>
<th>Relationship Between the Rate of Occurrence of OMN and BMN After Excluding Cigarette Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>$P$ value</td>
</tr>
<tr>
<td>control group</td>
<td>0.879</td>
<td>$&lt; 0.01$</td>
</tr>
<tr>
<td>low concentration</td>
<td>0.359</td>
<td>$&lt; 0.05$</td>
</tr>
<tr>
<td>contact group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>medium concentration</td>
<td>0.304</td>
<td>$&lt; 0.05$</td>
</tr>
<tr>
<td>contact group</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OMN: micronuclei in buccal mucosal cells; BMN: micronuclei in peripheral blood lymphocytes
DISCUSSION

ACN is a monomer widely used in organic syntheses and is an important chemical substance. At the same time, however, it is also an important environmental contaminant that imposes a latent threat to the health of both working and general populations. Animal experiments and human epidemiological studies reveal that ACN has carcinogenic activity in experimental animals [1]. The results of routine genetic toxicity experiments indicate that the mutagenic activity of ACN in somatic cells is relatively weak [2]. The rate of occurrence of micronucleus is an indicator of damage during the intermediary stage of chromosome mutation and the rate of occurrence of micronuclei in the cytoplasm can be used to evaluate the chromosome damaging effects of a substance [4]. Lymphocytes obtained from peripheral blood are frequently used in the micronucleus test. Buccal mucosal cells can be obtained easily without resorting to an invasive procedure and thus are expected to be used widely in future micronucleus tests.

In our present study, the method for determining the rate of occurrence of OMN was used for the first time for monitoring a population having occupational contact with acrylonitrile for possible genetic damage. Our results clearly indicate that the rate of occurrence of OMN in the low and medium concentration contact groups was significantly higher than that in the control group; the difference was statistically significant ($P < 0.05$), indicating that there was genetic damage to different degrees in the people in the ACN contact population. In contrast, the rate of occurrence of BMN in only the medium concentration contact group was found to be statistically higher than that of the control group ($P < 0.05$). This finding could be due to the fact that the mechanism of occurrence of micronuclei in buccal mucosal cells is different from that in peripheral blood lymphocytes. In buccal mucosal cells, the ACN molecule itself is mainly responsible for the occurrence of micronuclei, whereas in the lymphocytes, the metabolite of ACN is responsible. Therefore, OMN is more sensitive than BMN in revealing ACN-induced genetic damage. After excluding the presence of possible contaminating factors by regression analysis, it was found that the contact amount of ACN and the amount of cigarette smoking are the common influencing factors for the rate of occurrence of OMN and BMN. A correlation analysis revealed that there is a significant correlation between the rate of occurrence of OMN and that of BMN.

It is generally believed that the intermediary product of in vivo metabolism of ACN, i.e., epoxy acrylonitrile, is responsible for its genetic toxicity, but the question of whether the ACN molecule itself is genetically toxic remains unsettled. In our present study, the OMN are surely induced mainly by ACN itself, in that it is not
possible to completely metabolize ACN in the oral cavity and the rate of occurrence of OMN in the low concentration contact group is already statistically higher than that in the control group ($P < 0.05$). In the peripheral blood, most of ACN has been metabolized and thus the formation of micronuclei is mainly induced by the metabolite of ACN, thus the increase in the rate of occurrence of micronuclei is observable only in the medium concentration contact group. These findings suggest that the occurrence of micronuclei in the ACN contact population is related to the ACN molecule itself and may also account for the difference in the rank order of the partial regression coefficient of OMN and BMN.

Buccal mucosal cells represent the first line of defense against ACN intrusion and thus may be more susceptible to ACN-induced damage than the blood lymphocytes. Moreover, the OMN test may detect the occurrence of genetic damage in an earlier stage. The procedure involved in the OMN test is simple, convenient, time saving, economical, noninvasive, and easily acceptable by those being tested. Therefore, for monitoring a population, the OMN test may be used as a screening test for detecting genetic damage in populations that come into contact with acrylonitrile. However, the oral cavity is very vulnerable to various physical and chemical irritation effects. Habits and customary behavior such as cigarette smoking and the use of alcohol can also affect the rate of occurrence of OMN. Therefore, interference of various contaminating factors should be taken into consideration when evaluating the results of the OMN test in order to provide a more general and objective evaluation of the genetic effects of a toxic substance.

REFERENCES