RAS U-2

Request for Hearing and/or Petition to Intervene
In Re: Crow Butte Resources Request for License Renewal, Lic. No. SUA-1534
Docket No. 40-8943

***Via E-filing***

DOCKETED
USNRC

July 29, 2008 (8:15am)

OFFICE OF SECRETARY
RULEMAKINGS AND
ADJUDICATIONS STAFF

Office of the Secretary
U.S. Nuclear Regulatory Commission, 16th Floor
One White Flint North
11555 Rockville Pike
Rockville, MD 20852

**Petitioner:**
Oglala Sioux Tribe
By its Attorneys:
Elizabeth Lorina
Mario Gonzalez
Attorneys for the Oglala Sioux Tribe
522 7th Street, Suite 202
Rapid City, SD 57701
605-716-6355 x102 (telephone)
605-716-6357 (facsimile)
elorina@gnzlawfirm.com
gnzlaw@aol.com

Pursuant to 10 CFR Section 2.309, the undersigned petitioner states that it has an affected interest in this matter and desires to participate as a party and files this request for hearing and/or petition for leave to intervene and a specification of the contentions which should be litigated.

A hearing should be granted, and the undersigned should be entitled to participate in it if it has shown standing and has proposed at least one admissible contention that meets the requirements of Section 2.309(f).

This request was timely filed on July 28, 2008 via electronic filing. Capitalized terms that are not defined herein have the meanings assigned to them in the Application.



Template Secy-037

DS-03

**STANDING**

The Oglala Sioux Tribe ("Tribe") is the freely and democratically-elected government of the Oglala Sioux people, with a governing body duly recognized by the Secretary of Interior. As the duly recognized government of the Oglala Lakota people, the Tribe is authorized to act to protect the nation, and its people's, interests.

The Oglala Sioux Tribe is the successor-in-interest to the Oglala Band of the Teton Division of the Sioux Nation, and is a protectorate nation of the United States The Oglala Band reorganized in 1936 as the "Oglala Sioux Tribe of the Pine Ridge Indian Reservation" under section 16 of the Indian Reorganization Act of June 18, 1934, ch. 576, 48 Stat. 987, 25 U.S.C. § 476, and enjoys all of the rights and privileges guaranteed under its existing treaties with the United States in accordance with 25 U.S.C. § 478b.

The United States and the Oglala Band entered into a treaty of friendship and protection on July 5, 1825, 7 Stat. 252, which treaty was duly ratified by the United States and proclaimed on February 6, 1826. By Article 2 of the 1825 Treaty, the United States brought the Oglala Band and its members under its protection and the Oglala Band became a protectorate nation of the United States.

The United States and the seven bands of the Teton Division of the Sioux Nation, and others, entered into a treaty on September 17, 1851, 11 Stat. 749 ("1851 Fort Laramie Treaty" or "1851 Treaty"), which treaty was duly ratified by the United States. Article 5 of the 1851 Fort Laramie Treaty defined the territory of the bands of the Teton Division as follows ("1851 Treaty territory"):

> commencing the mouth of the White Earth River, on the Missouri River; thence in a southwesterly direction to the forks of the Platte River; thence up the north fork

> of the Platte River to a point known as the Red Bute, or where the road leaves the river; thence along the range of mountains known as the Black Hills, to the head-waters of the Heart River; thence down Heart River to its mouth; and thence down the Missouri River to the place of beginning.

In *Sioux Tribe v. United States*, 15 Ind. Cl. Comm. 577 (1965), the Indian Claims Commission ruled that the 1851 Treaty was a multi-lateral treaty by which the United States recognized the aboriginal territory of not only the seven Teton bands, but also the aboriginal territories of the other signatory tribes, including the Hidatsa, also known as the Gros-Ventre, the Mandan and the Arikara tribes. The Commission ruled that article 5 of the 1851 Treaty recognized the seven Teton bands' joint and several aboriginal Indian title to the entire sixty million acre area west of the Missouri River.

Unconsented encroachments on the 1851 Treaty territory by the United States and its citizens resulted in the Powder River War of 1866-1868 between the United States and the Teton bands. Peace was concluded between the United States and the Teton bands by treaty on April 29, 1868, 15 Stat. 635 ("1868 Fort Laramie Treaty" or "1868 Treaty"), which treaty was duly ratified by the United States on February 16, 1869 and proclaimed on February 24, 1869. The 1868 Treaty provided for a mutual demobilization without terms of surrender on either side.

Article 2 of the 1868 Treaty established a designated territory within the 1851 Treaty territory boundaries for the seven Teton bands and other Sioux bands. This territory is commonly referred to as the "Great Sioux Reservation," and is described in article 2 of the 1868 Treaty as follows:

> Commencing on the east bank of the Missouri River where the forty-sixth parallel of north latitude crosses the same, thence along low-water mark down said east

> bank to a point opposite where the northern line of the State of Nebraska strikes the river, thence west across said river, and along the northern line of Nebraska to the one hundred and fourth degree of longitude west from Greenwich, thence north on said meridian to a point where the forty-sixth parallel of north latitude intercepts the same, thence due east along said parallel to the place of the beginning; and in addition thereto, all existing reservations on the east bank of the said river shall be, and the same is, set apart for the absolute and undisturbed use and occupation of the Indians herein named . . . .

Article 12 of the 1868 Treaty further provided that no future cessions of territory within the Great Sioux Reservation would be of "any validity or force . . . unless executed and signed by at least three-fourths of all the adult male Indians, occupying or interested in the same . . . ." Under article 12, the United States and Teton bands agreed to limit their sovereign powers to cede and to accept cessions of land for the protection and peace of both parties.

By the Act of February 28, 1877, ch. 72, 19 Stat. 254 ("1877 Act"), Congress purported to ratify and confirm an agreement between commissioners on behalf of the United States and the Teton and other bands of the Sioux Nation and the Northern Cheyenne and Arapaho tribes. The purported agreement provided for the cession of over 7 million acres of territory in the western part of the Great Sioux Reservation, including the Black Hills. No such agreement existed in fact or in law.

The 1877 Act has the dubious distinction of being characterized as such: "A more ripe and rank case of dishonorable dealings will never, in all probability, be found in our history, which is not, taken as a whole, the disgrace it now pleases some persons to believe." United *States v. Sioux Nation of Indians*, 448 U.S. 371, 388 (U.S. 1980) (quoting *United States v. Sioux*

*Nation of Indians,* 207 Ct. Cl., 234, 241 (1975)). To coerce the Lakota into amending the 1868 Treaty, "[i]n August 1876, Congress enacted an appropriations bill providing that "hereafter there shall be no appropriation made for the subsistence" of the Sioux, unless they first relinquished their rights to the hunting grounds outside the reservation, ceded the Black Hills to the United States, and reached some accommodation with the Government that would be calculated to enable them to become self-supporting. Act of Aug. 15, 1876, 19 Stat. 176, 192." *Sioux Nation of Indians*, 448 U.S. at 381. This was also known as the "sell or starve campaign." Despite these efforts, only 10 per cent of the adult males signed. *Id.* at 383.

**Special Consideration due to the Oglala Sioux Tribe in these Proceedings**

As a federally recognized tribe, the Oglala Sioux Tribe has a trust relationship with the United States government, including all its agencies. *See e.g. Pueblo of Santa Ana v. United States,* 1997 U.S. Claims LEXIS 329 (Fed. Cl. Dec. 2, 1997) ("The federal government's fiduciary duty to Indian tribes applies to all federal agencies and programs.") *Parravano v. Babitt,* 70 F.3d 539 (9th Cir. 1995) ("This trust responsibility extends not just to the Interior Department, but attaches to the federal government as a whole."); *Nance v. EPA,* ("It is fairly clear that any Federal government action is subject to the United States' fiduciary responsibilities toward the Indian Tribes.").

The Court has recognized that the trust duty owed to Indian Tribes, who are "dependent and sometimes exploited people", is the highest legal duty.

> In carrying out its treaty obligations with the Indian tribes, the Government is something more than a mere contracting party. Under a humane and self imposed policy which has found expression in many acts of Congress and numerous decisions of this Court, it has charged itself with moral obligations of the highest responsibility and trust.

*Seminole Nation v. United States*, 316 U.S. 286, 296-297 (1942). Any action taken by the Nuclear Regulatory Commission, a federal agency, must be done with consideration of this duty

owed to the Tribe. It must be recognized that this duty is higher than that owed to the American people as a whole. As trustee for the Tribe, the NRC has an affirmative duty to ensure that any action it takes, including granting a renewal license, will not negatively impact the Tribe.

**The Oglala Sioux Tribe's Interest in the Renewal**

The Oglala Sioux Tribe has recognized aboriginal territory in the area of the renewal permit. Many of the cultural resources in the area are associated with the Tribe. Furthermore, The Pine Ridge Indian Reservation ("Reservation"), the location of the Tribe's land, is located only 30 miles from the Crow Butte site. Crow Butte's use of water for its in situ leach mining operation affects the ability of the Tribe and its members to use its water sources. The in situ leach mining not only depletes the aquifers used by the Tribe so that less water is available, but also negatively affects the quality of water. The Tribe will address its specific contentions with the renewal application below.

If the license renewal is granted, the Tribe will continue to be negatively and irreparably affected by the ISL mining operations. Since the mining began, the Tribe has experienced a loss in total available water, the water that is left is of lesser quality, and numerous negative health impacts to its people. It also impacts live stock using well water or the White River and its tributaries as their source of water.

## ENVIRONMENTAL CONTENTIONS

*Environmental Contention A- There is no evidence based science for the CBR's conclusion that ISL mining has "no non radiological health impacts" (See Table 8.6-1 of application), or that nonradiological impacts for possible excursions or spills are "small" (see 7.12.1 of application).*

**Issue of law** – NEPA requires that federal agencies prepare a "detailed statement" or environmental impact statement, for every major federal action "significantly affecting the quality of the human environment". 42 U.S.C § 4332(2)(C). In the licensing and regulatory

actions, the NRC is required to consider "the alternatives available for reducing or avoiding adverse environmental and other effects." 10 C.F.R §51.71 (d) (incorporated by reference in 10 C.F.R. 51.90).

**Basis for the contention -** CBR has provided no scientific evidence to show that residents in an around the mining site and up to Pine Ridge have no significant risk to their health. In a letter presented to the NRC in 1989, by a geologist John Petersen, his expert opinion showed the mining aquifer used by CBR likely communicated with the aquifers that supply the drinking water to the residents on the Pine Ridge Indian Reservation. Again in 2008, the issue of aquifer communication is raised by Dr. Hannan LaGarry, showing the likelihood of spills from the mining site could reach the residents of Pine Ridge. Based on this data, CBR must present data to show the alternatives, if any, that are available to reduce or avoid environmental and other effects **beyond** the confines of the mine itself. In 5.8.1.3 spill contingency plans of the renewal application, CBR outlines its plan to control for spills which could migrate into the aquifer that supplies drinking water. In its plan, CBR indicates monitoring wells are placed within 300 feet of the mine and monitored "biweekly". However, the plan does not recognize that leaks could occur and go undetected if the scheduled testing did not coincide with a leak. Furthermore, the plan at 5.8.8.2 Ground Water Monitoring, does not indicate that the monitoring wells are tested for uranium whether radioactive or depleted, and other heavy metals known to be toxic and linked to the development of cancer if ingested over time. There is no scientific basis for excluding uranium from the monitor well testing.[1] In the report submitted by Richard Abitz, he states: "As uranium is mobilized and transported by the high oxygen and alkalinity in the lixiviant, there is no valid scientific reason to exclude it from the list of excursion monitoring parameters." Dr. Abitz states further: "Uranium is a key indicator of lixiviant excursion because

[1] See expert report submitted by Dr. Richard Abitz.

its concentration in baseline wells is generally two or three orders of magnitude lower than the lixiviant...there is no rational basis to exclude the best excursion indicator...". CBR also fails to indicate where the lab testing of the wells is done, whether the monitoring wells are tested by its own staff or independent outside lab. The Oglala Sioux Tribe must be assured that any testing is accurate and unbiased. Given the price of uranium around $135/pound, and potential gross profits at CBR of $50 mil a year, any appearance of impropriety must be addressed.

CBR has failed to produce any scientific data to substantiate their finding of "no non radiological health effect". In fact CBR outlines ecological impacts starting at page 7 – 17, and through 7 – 23 addressing the issues of the effects of ISL mining on wetlands, wildlife and fisheries, small mammals and birds, big game mammals, upland game birds, sharp-tailed Grouse, raptors, fish and macroinvertibrates, endangered species, swift fox, bald eagle, black footed ferret, whooping crane, and reptiles and only devotes one paragraph in 7.12.1 of its application to the non radiologic impacts of ISL mining on humans. Furthermore, there is no literature review to show that CBR made an effort to find new scientific data on the non radiological impact of ISL mining on the public at large and the Oglala Sioux Tribe specifically. Also CBR does not outline in its application the possible health hazards of ingesting drinking water that is contaminated with uranium.

**Issued raised in this contention are within the scope of this proceeding** – The contentions petitioners raise is within the scope of the proceeding because the contentions are with the application for source material license renewal. In 10 C.F.R 40.32(c)(d) General Requirements for Specific License it states: (c) The applicant's proposed equipment, facilities and procedures are adequate to protect health and minimize danger to life or property; and (d) The issuance of the license will not be inimical to the common defense and security or to the health and safety of

the public. Additionally, the accuracy and completeness of the application is called into question under 10 C.F.R 40.9 (a) and(b) Domestic Licensing of Source Material General Provisions for completeness and accuracy of information provided on the application for licensure. Under (a) the applicant or licensee is required to provide information that "is complete and accurate in all material respects". And under (b) the applicant or license shall notify the Commission of information identified by the applicant or licensee as having for the regulated activity a significant implication for the public health and safety or common defense and security.

**The issue raised in this contention is material to the findings of the NRC of whether CBR should be granted a renewal of its license for source material -** The law is clear under NEPA that the NRC must produce a "specific statement" in a major federal action, such as granting a license renewal, where there is a significant impact on the human environment. 42 U.S.C. § 4332(2)(C). Given the Trustee relationship of the NRC to the Oglala Sioux Tribe, health and safety should be material to and have the highest priority in consideration in these license renewal proceeding. The issues of accuracy of information provided on the application particularly where the information is related to the health and safety of the Oglala Sioux Tribe is also material to the finding of whether to issue a renewal permit for CBR's source mining license.

**Statement of facts and opinions** – Current scientific literature: In an NIH funded study published in 2005, Diane Stearns, PhD and her research team at Northern Arizona University, found a direct correlation between exposure to depleted uranium and mutations in cells.[2] Dr. Stearns posits from her findings that "health risks for uranium exposure could go beyond those

[2] Uranyl acetate induces hprt mutations and uranium-DNA adduct in Chinese hamster ovary EM9 cells. Diane M. Stearns et. al. Mutagenesis vol. 20 no. 6 pp. 417 – 423, 2005.

for radiation exposure".[3] The basis of her study was the interest in environmental exposure risks to uranium in drinking water. Stearns identifies insufficiency in the study of environmental uranium exposure and Native American populations other than miners.[4] Until the findings of the Stearns' study were published, it was thought that only radiation exposure from uranium caused the risk for cancers and other health problems. Stearns found that both the radioactivity of uranium, and the non radiological active form of uranium caused cell mutations. Dr. Stearns concluded: "This possibility of direct U-DNA interaction should be considered when extrapolating potential risks for people exposed to uranium in the absence of measurable radioactivity, for example in soil and drinking water, and in DU – containing shrapnel."[5]

Recent research includes a study published in 2007 through the NIH, by Stefanie Raymond-Whish et al. from the Department of Biological Sciences at Northern Arizona University, and the College of Medicine at the University of Arizona. The paper addresses drinking water containing uranium and concludes that populations exposed to uranium, even at levels outlined by the EPA, should be followed for increased risk of fertility problems and reproductive cancers.[6] The Raymond-Whish study on drinking water also recognized the impact of heavy metals, many of which are byproducts of uranium mining as exhibiting properties that stimulate proliferation of breast cancer cells.[7]. Raymond -Whish used drinking water with uranium levels that were within the EPA standards of 30 μg/L and within the measured drinking water samples from numerous water resource samples on the Navajo Indian Reservation.[8]

The NRC must not ignore the connection between higher cancer rates at Pine Ridge

---

[3] Id. At 417.
[4] Id.
[5] Id. At 421.
[6] Drinking Water with Uranium below the US EPA Water Standard Causes Estrogen Receptor-Dependant Responses In Female Mice. Stefanie Raymond-Whish et. Al., Environmental Health Perspectives. Vol 115 No. 12 Dec 2007.
[7] Id. At 1711.
[8] Id at 1714.

Reservation and current practices of ISL mining that produce cancer causing agents. Recent research on cancer rates specifically concerning the Oglala Sioux Tribe, is found in a study published in the American Journal of Public Health 2005 by Deborah Rogers and Daniel Petereit. The study reviews the high cancer mortality rates for 3 western South Dakota tribes including the Oglala Sioux Tribe.[9] In their study funded by the National Cancer Institute, Rogers and Petereit cite data from the Indian Health Services based on reports for the years 1994 and 1998.[10] These tribes had cancer mortality rates an astounding 40% higher than the national averages.[11] Lung cancer mortality was 62% higher than the national average, and colorectal cancer mortality was 58% higher than the national average.[12] One of the leading causes of death was malignant neoplasm at a rate 15% higher than the national average.[13] More recently in a report of cancer rates to the National Cancer Institute, American Indians have shown high rates of cancer, despite the overall decline of cancer nationally.[14] Specifically in the northern plains, cancer rates for lung, colon uterine, kidney, and non hodgkin lymphoma were between 2% to nearly 50% higher than the national average.[15]

Native Americans have a long history of the US government inadequately addressing the health impacts from poorly managed uranium mining facilities. On October 23,2007, Doug Brugge, PhD, MS a Harvard educated Developmental Biologist and Industrial Hygienist, gave testimony on the inadequacy of the government response to Native American health issues

---

[9] Cancer Disparities Research Partnership in Lakota Country: Clinical Trials, Patient Services, and Community Education for the Oglala, Rosebud, and Cheyenne River Sioux Tribes. Deborah Rogers, MS, and Daniel Peteriet, MD. American Journal of Public Health. Pages 2129 – 2132. Vol.95, No.12, December 2005.
[10] Id at 2129
[11] Id.
[12] Id.
[13] Id.
[14] Annual Report to the Nation on the Status of Cancer, 1975 – 2004, Featuring cancer in American Indians and Alaska Natives. David K. Espey, MD et. Al. Cancer. Vol 110 Issue 10 Pages 2119 – 2152. Oct 15, 2007.
[15] See supplemental Tables 7 – 8.

arising from government approved uranium mining.[16] He emphasized the inadequacies in three ways: 1. Delay in providing timely compensation to uranium mine workers. Dr. Brugge testified that it took over 4 decades of testimony to Congress before Navajo miners were given some semblance of compensation for their injuries. 2. Lack of appropriate health impact studies on the effects of uranium mining in the Native American communities. In his testimony before Congress, Dr. Brugge states that despite a clear showing of a connection of uranium and heavy metals to the development of cancer and birth defects, the only study on health impact is for kidney function. 3. The disparity in treatment between Indian and Non-Indian communities following a major radioactive waste spill. Dr. Brugge referred to Church Rock, New Mexico, where the largest radioactive waste spill in US history occurred, and testified about how this was treated in comparison to the incident at Three Mile Island (TMI). Dr. Brugge stated: "This release, which was substantially larger than the release at TMI, flowed into a low-income, largely Native American community. This incident has been virtually ignored in the press and scientific literature."

Given the high rates of death from cancers, and the historical treatment of American Indians regarding health effects of exposure to toxins from uranium mining by the US Government, the issues of health and safety must to be addressed by the NRC with specificity. The NRC has an opportunity that is within their scope of power to insure that ISL proposed mining practices meet the standard to insure safety in a preventive manner. The NRC must not ignore current scientific evidence that suggests that acceptance of prior health impact statements in applications are now grossly inadequate and not specific. The health impacts of ISL mining must be addressed as a requirement for a license renewal application and should include the

[16] See testimony by Dr. Brugges to the Committee on Oversight and Government Reform Congress of the United States House of Representatives. October 23, 2007.

impact of both radioactive and non radioactive uranium in drinking water of the Oglala Sioux Tribe at Pine Ridge Indian Reservation. It is the legal responsibility of the NRC as Trustee and protector of the health and safety of the Oglala Sioux Tribe, to require, with specificity, a statement of the effects on the human environment prior to granting a renewal license to CBR.

**Environmental Contention B**

*The Oglala Sioux Tribe has not been consulted with regarding the cultural resources that may be in the license renewal area. The Applicant has identified what it believes to be cultural resources in the area, but the Tribe has had no input on this list, and it therefore cannot be complete. Furthermore, the Applicant has provided that it will work in conjunction with the Nebraska State Historical Society to avoid the identified resources, but this ignores mandated participation of the Oglala Sioux Tribe.*

**Issue of fact or law-** The Tribe must be consulted with regarding any cultural resources in the area whenever there is major federal action, i.e. the NRC granting a mining permit to the Applicant. NHPA 16 U.S.C. 470 *et.seq.*

**Basis of Contention -** It is undisputed that the Crow Butte area is part of the 1851 Treaty area, which recognized such area as the aboriginal land of the Teton Sioux Nation, including the Oglala Lakota people. Therefore, any Indian sites or artifacts in the area would be connected to the Tribe. CBR is not qualified to determine whether cultural sites or artifacts or sites even exist, or how to preserve them. These potential artifacts and evidence are from *Oglala* and *Lakota* history, and no body or entity is more qualified to judge their existence or importance that the Oglala Lakota Oyate (people) themselves- which is precisely why consultation is required and those determinations are not left to the federal agency or company proposing action.

**Issued raised in this contention are within the scope of this proceeding** – It is within the scope because the NRC, under NEPA, is mandated to take a "take a 'hard look' at the environmental consequences" of a major federal action before taking that action. *Mid States Coalition for Progress v. Surface Transp. Bd.*, 345 F.3d 520, 533 (8th Cir. 2003) (internal

citations omitted). NEPA further requires that federal agencies prepare an environmental impact statement (EIS) when "major Federal actions significantly affecting the quality of the human environment[.]" 42 U.S.C. § 4332(2)(C). The NRC must take the potential cultural resources at the license site into consideration when deciding whether to grant a license.

**The issue raised in this contention is material to the findings of the NRC of whether CBR should be granted a renewal of its license for source material -** This contention puts forth the obligations the NRC has obligations as a federal agency under federal law. As federally recognized tribe, the Tribe has a right of consultation when there is major federal action that affects its interests. By granting this license renewal, NRC would be taking major federal action. The National Environmental Protection Act, NEPA, guarantees a right of consultation to Indian tribes when there is major federal action. NEPA mandates that the government "preserve important historic, cultural, and natural aspects of our national heritage, and maintain, wherever possible, an environment which supports diversity, and variety of individual choice." 42 USC § 4331(b). NEPA then triggers the National Historic Preservation Act (NHPA), 16 U.S.C.S. § 470f., Native American Graves Repatriation Act (NAGPRA), 25 U.S.C. 3001 et seq. and the Archaeological Resources Protection Act, 16 U.S.C. 4700 *et seq.* Federal agencies are required to consult with federally recognized Indian tribes that may attach religious or cultural significance to the project area, even if the project area is not within its reservation under Section 106 of the NHPA. 36 C.F.R. 800.4(f)(2).The federal agency is further required to consult with a tribe's Tribal Historic Preservation Officer (THPO) if there is one, and a tribal representative if not.

**Facts to support this contention include -** The Oglala Sioux Tribe is a federally recognized tribe, entitled to all the rights under federal law that such tribes are entitled to, including

consultation under Section 106 of NHPA, as well as the obligations owed to it from its trustee, the federal government. Furthermore, the Crow Butte area is within the 1851 Treaty area, which is recognized as the aboriginal land of the Tribe, and therefore the Tribe ascribes cultural and religious significance to many sites in that area. The Tribe has its own THPO, who should be consulted before determining that there are no significant cultural resources in the area. The NRC cannot make that determination without consultation with the Tribe. The Application itself states that it will work with the *Nebraska State Historical Society* to avoid the identified cultural resources, including Native American ones, and ignores the Tribe's right to participate. The Application also states at Section 4.8 that the Nebraska SHPO has determined that the identified sites or artifacts are not eligible for inclusion on the National Register, but the Tribe has not been consulted with regarding any sites or potential sites.

**Specific examples from the Application -** CBR's Renewal Petition offers Table 2.4-1, which details the cultural resources in the area. However, the Tribe has neither had the opportunity to evaluate the completeness of this list, nor the opportunity to evaluate the accuracy of the significance ascribed to the items on the list. Section 7.8 of the Application provides that "[a]ny further construction activities will avoid these identified resources and coordination will be maintained with the Nebraska State Historical Society." Further, Section 4.8 asserts that the Nebraska SHPO has determined that the cultural resources are not eligible for inclusion on the National Register, but no consultation has been done with the Tribe. However, this ignores the participation of the Tribe for these identified Native American cultural resources. The participation of state agencies is not adequate under NEPA.

**Environmental Contention C**

*In 7.4.2.2 in its application for renewal, CBR characterization that the impact of surface waters from an accident is "..minimal since there are no nearby surface water features." does not accurately address the potential for environmental harm to the White River.*

**Issue of law** – NEPA requires that federal agencies prepare a "detailed statement" or environmental impact statement, for every major federal action "significantly affecting the quality of the human environment". 42 U.S.C § 4332(2)(C). In the licensing and regulatory actions, the NRC is required to consider "the alternatives available for reducing or avoiding adverse environmental and other effects." 10 C.F.R §51.71 (d) (incorporated by reference in 10 C.F.R. 51.90).

**Basis for Contention** – In 7.4.2.2 on its application, CBR ignores the White River as a potential surface water that is affected in the event of an accident. CBR contradicts their own claim that there is no surface water that would be affected in the event of an accident, by identifying in 7.5.4 of the application that Squaw Creek and English Creek lie within the area of the license, and that they are small tributaries of a "major regional watercourse, the White River".[17] In Dr. LaGarry's expert opinion, the White River alluvium, can receive contaminants from three sources 1) from surface spills at the Crow Butte mine site 2) from water transmitted through the Chamberlain Pass Formation where it is exposed at the land surface, and 3) through faults.[18]

**Issued raised is within the scope of the proceeding** – The purpose of the proceeding is to determine whether or not to grant a renewal license to CBR. Therefore information contained in its application is within the scope, both in terms of accuracy, and specific impact of ISL mining on the environment. In 10 C.F.R 40.32(c)(d) General Requirements for Specific License it states: (c) The applicant's proposed equipment, facilities and procedures are adequate to protect health and minimize danger to life or property; and (d) The issuance of the license will not be inimical

[17] See 7.5.4 of CBR application for renewal.
[18] See page 3 of Dr. Hanna LaGarry report.

to the common defense and security or to the health and safety of the public. Additionally, the accuracy and completeness of the application is called into question under 10 C.F.R 40.9 (a) and(b) Domestic Licensing of Source Material General Provisions for completeness and accuracy of information provided on the application for licensure. Under (a) the applicant or licensee is required to provide information that "is complete and accurate in all material respects". And under (b) the applicant or license shall notify the Commission of information identified by the applicant or licensee as having for the regulated activity a significant implication for the public health and safety or common defense and security.

**The issue raised in this contention is material to the findings of the NRC of whether CBR should be granted a renewal of its license for source material. -** The law is clear under NEPA that the NRC must produce a "specific statement" in a major federal action, such as granting a license renewal, where there is a significant impact on the human environment. Given the Trustee relationship of the NRC to the Oglala Sioux Tribe, environmental protection should be material and have the highest priority in consideration in this license renewal proceeding. The issues of accuracy of information provided on the application particularly where the information is related to the possible harm to the environment of the Oglala Sioux Tribe is also material to the finding of whether to issue a renewal permit for CBR's source mining license.

**Statement of facts and opinions** – In their expert reports, Paul Ivancie, W. Austin Creswell, and Dr. Hannan LaGarry agree that the White River alluvium should be evaluated for extent of contamination. They recognize the White River alluvium as a potential pathway for contamination, which directly contradicts CBR's characterization of impact from an accident as "..minimal since there are no nearby surface water features."[19] CBR provides no scientific data to support its claim that an accident would have no impact on surface waters of the White River.

[19] See 7.4.2.2 in application for license renewal.

The law is clear under both NEPA the NRC has a duty to provide "specific statement" regarding the effects of a major federal decision, such as licensing of source material, where it has substantial affect on the human environment. Additionally the NRC is required by 10 C.F.R §51.71 (d) (incorporated by reference in 10 C.F.R. 51.90) to provide the alternatives available for reducing or avoiding adverse environmental and other effects.

As Trustees of the Oglala Sioux Tribe, the NRC has a legal duty to protect the water and environment from harm. Since the White River runs through the Pine Ridge Reservation, and there is scientific evidence of the potential and actual contamination (documented excursions and leaks), the NRC must require a specific statement of the potential and current damage of the White River prior to granting a renewal of CBR's license. Additionally, it is within the scope of this proceeding to require additional information on contamination, and respond to scientific data showing the need for water sampling **beyond** the mining site, and in particular along the White River prior to granting a license renewal.

**Environmental Contention-D**

*In 7.4.3 CBR's Application incorrectly states there is no communication among the aquifers, when in fact, the Basal Chadron aquifer, where mining occurs, and the aquifer, which provides drinking water to the Pine Ridge Indian Reservation, communicate with each other, resulting in the possibility of contamination of the potable water.*

**Issue of law** – NEPA requires that federal agencies prepare a "detailed statement" or environmental impact statement, for every major federal action "significantly affecting the quality of the human environment". 42 U.S.C § 4332(2)(C). In the licensing and regulatory actions, the NRC is required to consider "the alternatives available for reducing or avoiding adverse environmental and other effects." 10 C.F.R §51.71 (d) (incorporated by reference in 10 C.F.R. 51.90).

**Basis for Contention-** CBR's Application uses its assertion that the aquifers do not communicate to support its contention that the ISL mining is not a threat to the water sources of the surrounding areas. If the aquifers do communicate, whether by faults or other means, that calls into question the true safety of this method.

**Issued raised in this contention are within the scope of this proceeding -** Pursuant to 10 CFR 40.9, information provided by the applicant must be complete and accurate in all respects. The Tribe contends that the information provided by the Applicant in its application are neither complete nor accurate. CBR provides that

**The issue raised in this contention is material to the findings of the NRC of whether CBR should be granted a renewal of its license for source material.** The contentions petitioners raise is within the scope of the proceeding because the contentions are with the application for source material license renewal. In 10 C.F.R 40.32(c)(d) General Requirements for Specific License it states: (c) The applicant's proposed equipment, facilities and procedures are adequate to protect health and minimize danger to life or property; and (d) The issuance of the license will not be inimical to the common defense and security or to the health and safety of the public. Additionally, the accuracy and completeness of the application is called into question under 10 C.F.R 40.9 (a) and(b) Domestic Licensing of Source Material General Provisions for completeness and accuracy of information provided on the application for licensure. Under (a) the applicant or licensee is required to provide information that "is complete and accurate in all material respects". And under (b) the applicant or license shall notify the Commission of information identified by the applicant or licensee as having for the regulated activity a significant implication for the public health and safety or common defense and security.

As a federal agency, the NRC has an affirmative obligation to apply federal law in its actions. In this instance, not only is the NRC required to act consistent with its trust responsibility to the Tribe, but further apply the *Winters* doctrine in its decision-making process when ruling on CBR's application. As a trustee to the Tribe, the NRC must not act in a way that damages the interests of the Tribe. The Tribe needs sufficient quantities of clean water to carry out its ranching and agricultural pursuits, as well as provide clean, safe water for its people.

**Facts to support this contention include -**

a.. The aquifers do communicate, and therefore there is the potential for contamination of the Pine Ridge water supply by CBR's activities. See, Expert report of Dr. LaGarry:

> Also, many of the ancient river deposits of the Arikaree and Ogallala Groups, along with the alluvium deposited by modern rivers, follow the faults zones because fractured rock erodes more easily. Swinehart & others (1985) and Diffendal (1994) reported faults that could transmit contaminants from Crawford to Chadron, and from Crawford to Pine Ridge, South Dakota. In its license amendment for the North trend expansion, Crow Butte Resources reports a fault along the White River that could transport contaminants from the ISL mine to the White River, and from the river directly to Pine Ridge, South Dakota.

b. The Reservation already has severe problems with adequate potable water to meet the needs of its residents. Continued and increased mining of these communicative aquifers poses a serious health and safety risk to the residents of the Reservation.

**Genuine Dispute Exists with the Application on a Material Fact or Issue of Law-** In Section 7.4.3 of the Application, it states that

> Because the Basal Chadron Sandstone (production zone) is a deep confined aquifer, no surface water impacts are expected. Further, the geologic and hydrologic data presented in Sections 2.6 and 2.7, respectively, demonstrate that (1)the occurance of uranium mineralization is limited to the Basal Chadron Sandstone; and (2) the Basal Chadron is isolated from underlying and overlying sands. Hence, the mining operations are expected to impact water quality only in the Basal Chadron Sandstone, and restoration operations will be conducted in the Basal Chadron following completion of mining.

The November 8, 2007 letter from the NDEQ to CBR questioned CBR's assertion that there was no hydraulic communication between the Basal Chadron Sandstone and the White River as lacking scientific support. The communication among the aquifers, including those leading to the Reservation, are supported by Dr. LaGarry's expert report. CBR incorrectly states in 7.12.4.2 that "[t]he solutions in the zone to be mined will be controlled and adequately monitored to ensure that migration does not occur. CBR's application fails to disclose the NDEQ's November 8, 2007 critique of its proposal for the North Trend Expansion Area".

**Issue of law or fact-** CBR, as identified by Dr. Fischbein and other NDEQ scientists, has failed to account for the White River Fault/Fold that is present in the southern portion of the North Trend Expansion Area ("NTEA"), which may affect the control of any migrations outside the mining area.

**Environmental Contention-E**

*CBR's application incorrectly states in 7.11 that "Wastes generated by the facility are contained and eventually removed to disposal elsewhere."*

**Issue of law or fact-** In May 2008, the Nebraska Department of Environmental Quality ("NDEQ") filed a complaint against CBR in the District Court of Lancaster County, CI08-2248, for violating the NDEQ-issued Underground Injection Control Permit, because CBR released well development water upon the surface of the ground during CBR's well development and drilling process. This occurred daily from July 1, 2003 until March 31, 2006. The parties entered into a consent decree on May 28, 2008, and fines were levied against CBR.

**Basis for Contention-** The Application states that all generated waste is contained and disposed elsewhere, but it is known that CBR has not always complied with this requirement.

This contention is within the scope of this proceeding. 10 CFR 40.32c provides that the application will be approved if "[t]he applicant's proposed equipment, facilities and procedures are adequate to protect health and minimize danger to life or property". CBR has disposed of waste water in a manner that is inconsistent with the requirements for its application. Because CBR's procedures demonstrably do *not* protect health, nor does it minimize danger to life or property, it clearly does not meet the requirements for license renewal. furthermore, since waste water was simply dumped on the ground, instead of being properly contained and disposed of, as CBR asserts in its application, it does not meet the requirements for application accuracy under 10 CFR 49.1 A and B.

**Issued raised in this contention are within the scope of this proceeding-**The NRC must find that the procedures provided by CBR in its application are adequate to protect health and safety and that the application submitted is complete and accurate in all respects.

**The issue raised in this contention is material to the findings of the NRC of whether CBR should be granted a renewal of its license for source material-** CBR is known to have previously dumped waste on the ground, and not properly disposed of it as it is required to do.

Intervention Requested

Intervention is requested, in addition to a request for a hearing. If the petition for leave to intervene as a matter of right is denied, then this request includes a request to be allowed discretionary intervention. Further, regardless of the above, the Oglala Sioux Tribe has a right to participate under 10 CFR 2.315(c) as an affected, federally recognized Indian tribe.

Service on Licensee Applicant

A copy of this petition was mailed to:

Crow Butte Resources, Inc.
141 Union Blvd., Ste 330
Lakewood, CO 80228
Attn: Stephen Collings, President

Respectfully submitted this 28th day of July, 2008.

/s/ Elizabeth Lorina
/s/ Mario Gonzalez
Attorneys for the Oglala Sioux Tribe
522 7th Street, Suite 202
Rapid City, SD 57701
605-716-6355 x102 (telephone)
605-716-6357 (facsimile)
elorina@gnzlawfirm.com
gnzlaw@aol.com

Exhibit A- Copies of Referenced Studies and Reports

1. Testimony before the Committee on Oversight and Government Reform, Congress of the United States, House of Representatives, October 23, 2007, By Doug Brugge, PhD, MS.
2. "Cancer Disparities Research Partnership in Lakota Country", by Deborah Rogers, MS, and Daniel G. Petereit, MD, American Journal of Public Health, December 2005, Vol. 95, No. 12
3. "Uranyl acetate induces *hprt* mutations and uranium- DNA adducts in Chinese hamster ovary EM9 cells", by Diane M. Stearns, et al.
4. "Drinking Water with Uranium below the U.S. EPA Water Standard Causes Estrogen Receptor-Dependent Responses in Female Mice", by Stephanie Raymond-Whish, et al., Environmental Health Perspectives, December 2007, Vol. 115, No. 12

Exhibit B- Dr. Hannan Lagarry's Expert Report

**Exhibit A- Copies of Referenced Studies and Reports**

1. Testimony before the Committee on Oversight and Government Reform, Congress of the United States, House of Representatives, October 23, 2007, By Doug Brugge, PhD, MS.
2. "Cancer Disparities Research Partnership in Lakota Country", by Deborah Rogers, MS, and Daniel G. Petereit, MD, American Journal of Public Health, December 2005, Vol. 95, No. 12
3. "Uranyl acetate induces *hprt* mutations and uranium- DNA adducts in Chinese hamster ovary EM9 cells", by Diane M. Stearns, et al.
4. "Drinking Water with Uranium below the U.S. EPA Water Standard Causes Estrogen Receptor-Dependent Responses in Female Mice", by Stephanie Raymond-Whish, et al., Environmental Health Perspectives, December 2007, Vol. 115, No. 12

**Testimony before the Committee on Oversight and Government Reform**

**Congress of the United States**

**House of Representatives**

October 23, 2007

By Doug Brugge, PhD, MS

Good morning/afternoon Chairman Waxman and members of the committee. My name is Doug Brugge, I have a PhD in cellular and developmental biology from Harvard University and an MS in industrial hygiene from the Harvard School of Public Health. I am currently associate professor in the department of public health and family medicine at Tufts University School of Medicine. I also direct the Tufts Community Research Center. I have over 20 academic publications about uranium and the Navajo people, including a 2006 book that I co-authored, entitled The Navajo People and Uranium Mining. I have studied the Navajo people in part because they are facing a crisis in uranium contamination.

Appearing before this congressional hearing today reminds me of the long history of such hearings, beginning in the 1960s and continuing through the 1970s, 80s and 90s, that sought and eventually achieved a semblance of compensation for Navajo and other uranium miners. I am deeply saddened by the fact that so little has been accomplished over those decades to eliminate the health hazards faced by the enormous quantities of uranium waste on the Navajo reservation. There has been too little research on the health impacts of uranium mining in Navajo communities. The one study underway, for example, will mostly address kidney disease and not birth defects or cancer. Today as we begin the public process of addressing community exposures, I can only hope that the path is far shorter than the one traveled by the uranium miners and their families.

I will now spend a few moments describing the hazards faced by the Navajos today. Clearly, uranium ore is a toxic brew of numerous nasty hazardous materials. Uranium, itself highly toxic, gives rise to a series of other radioactive decay elements that are found in raw, natural ore. Most significant among these are radium and thorium, both of which are highly radioactive. When radium decays it produces radon gas, a potent toxicant. Because it is a gas that becomes

airborne, when radon decays it transforms into a series of highly radioactive "radon daughters" that can lodge in the lungs.

The primary heavy metal toxicants in uranium ore include uranium itself and arsenic, as well as vanadium and manganese. During the first phase of processing uranium, most of the uranium is removed, leaving behind mill tailings which retain most of the other toxic contaminants from the ore. The milling of uranium is an industrial process that involves crushing and grinding of the rock and the addition of acids and organic solvents to facilitate concentration and removal of the uranium. Hence, uranium mill tailings and mill tailings effluent are not only highly radioactive, but they are acutely hazardous.

The health effects of uranium and its associated radioactive decay products and heavy metals that rise to the level of proven or near-proven causal links include:

1) Radon, which causes lung cancer and in fact, it is the primary source of lung cancer among Navajo uranium miners;
2) Uranium, which as a heavy metal causes damage to the kidneys and birth defects ;
3) Radium, which causes bone cancer, cancer of the nasal sinuses and mastoid air cells and leukemia; and
4) Arsenic, which causes lung and skin cancer, as well as neurotoxicity, hyperpigmentation and hyperkeratosis of the skin.

There are may also be many other negative health effects from exposure to uranium and its byproducts. In short, there is a clear causal link between uranium exposure and human health. The Navajos continually exposed to uranium and its byproducts even today face grave threats to their health.

I would like to conclude with some observations about the Navajo community of Church Rock, both historical and present day. Church Rock is located outside of Gallup, New Mexico, in the Navajo Nation. The Church Rock tailings spill remains the largest industrial release of radioactive wastes in the history of the United States. In 1979, only months after the Three Mile

Island release, a dam holding back a tailings lagoon maintained by United Nuclear Corporation failed, sending 94 million gallons of radioactive and acidic wastewater and 1,100 tons of toxic and radioactive mill waste into the Puerco River. This release, which was substantially larger than the release at TMI, flowed into a low-income, largely Native American community. This incident has been virtually ignored in the press and scientific literature.

For the people in Church Rock and other Navajo communities contaminated for decades with uranium ore tailings there are no "good" options, too much harm has already been done. But there are ways that we can gradually make things better so that maybe the children and the grandchildren of the Navajo uranium miners are not still grappling with this toxic legacy. A good start would be to provide sufficient resources to secure or remove contamination at these hazardous waste sites and to do so in a manner that prevents additional exposure to nearby residents. And Congress must fund the Navajo Nation and federal health agencies to provide resources for health studies among the tens of thousands of Navajo community members who still live next to abandoned mines and-or who were exposed to uranium from the contaminated dusts brought home by their working relatives.

I leave you to ponder a simple observation about this egregious situation: As terrible as the health effects that we know arise from toxins in uranium tailings, there are almost certainly additional ways that the health of Navajo people living near uranium mill and mine waste has been affected. If we are to understand the full extent of this injustice, we will also need additional health studies.

# Cancer Disparities Research Partnership in Lakota Country: Clinical Trials, Patient Services, and Community Education for the Oglala, Rosebud, and Cheyenne River Sioux Tribes

Deborah Rogers, MS, and Daniel G. Petereit, MD

Native Americans served by the Aberdeen, Billings, and Bemidji areas of the Indian Health Service (IHS) have a cancer mortality rate approximately 40% higher than that of the overall US population. The National Cancer Institute has funded Rapid City Regional Hospital to provide clinical trials, behavioral research, a genetic protocol, patient navigator services (assisting patients with health care coordination and financial issues and helping them to understand their options), and community education for members of 3 western South Dakota tribes.

Challenges faced by the project included obtaining multiple approvals from 3 tribes, 4 IHS facilities, and 5 institutional review boards; travel distances; lack of screening; red tape of referrals; and refusal by some payers to cover clinical trials. Building trust through ongoing communication and community presence is key to a successful project.

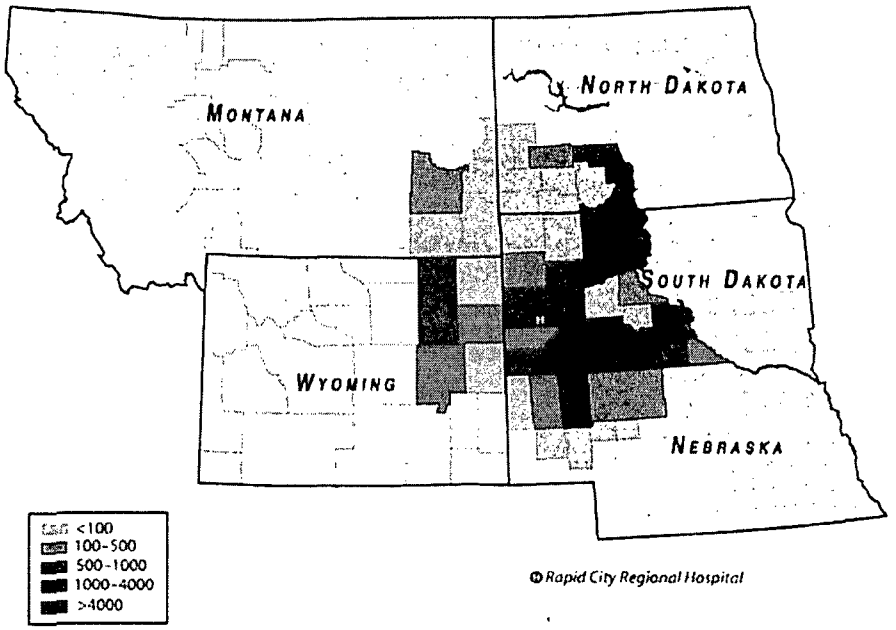
**IN THE UNITED STATES,** various subpopulations experience different rates of cancer detection, treatment, participation in clinical trials, and outcomes.[1–4] In particular, the population of Native Americans served by the 10-state Billings, Aberdeen, and Bemidji service areas of the Indian Health Service (IHS) suffers from a cancer mortality rate approximately 40% higher than that of the overall US population.[5] Researchers from the IHS analyzed cancer mortality data from the death certificate database of the National Center for Health Statistics, which were adjusted for racial miscategorization and the age structure of the population, and then summarized the results for 1994 through 1998. Although their rates of breast cancer mortality were approximately 15% lower than for Whites, Native Americans in this 10-state Northern Plains region had significantly higher average annual age-adjusted mortality rates for colorectal cancer (58% higher), lung cancer (62%), cervical cancer (79%), and prostate cancer (49%).[5]

Rapid City Regional Hospital, in the Black Hills of western South Dakota, provides secondary and tertiary cancer care for an estimated 60 000 Native Americans living within a 200-mile radius of Rapid City (Figure 1). Most of these tribal members live in the IHS Aberdeen Area (North Dakota, South Dakota, Iowa, and Nebraska). In 1996 through 1998, the life expectancy at birth (both genders) for Native Americans in the Aberdeen Area was 65.4 years, compared with 70.6 years for all IHS areas (1996–1998) and 76.5 years for the general US population (1997).[6]

The local population served by Rapid City Regional Hospital is growing very rapidly, with nearly 50% of the population aged younger than 18 years, according to data from the 2000 US Census. In 1996 through 1998, the leading causes of death in the Aberdeen Area were diseases of the heart (21% of deaths), malignant neoplasms (15%), unintentional injuries (14%), diabetes mellitus (8%), and chronic liver disease and cirrhosis (6%).[6]

A retrospective chart review was performed for 93 Native American radiation therapy patients treated at the hospital's Cancer Care Institute between January 1998 and October 2002.[7] The median one-way distance patients traveled was 109 miles (ranging from 5 to 215 miles). Thirty-seven percent of Native American patients traveled at least 150 miles each way. Of 61 Native American patients treated with curative intent, 28% had treatment delays (i.e., missed days) of 6 or more days and 15% had delays of 11 or more days. Thirty of the patients (half) experienced grade 2 radiation treatment toxicities, and 10 had grade 3 radiation treatment toxicities.

Statistics from Rapid City Regional Hospital's Tumor Registry (1990–2000) indicate that Native Americans are more likely than other patients to present with advanced (stage III or IV) disease, which leads to lower survival rates (Table 1).[7] For colorectal, breast, prostate, cervical, and lung cancer, approximately 50% of Native Americans arrived at the hospital with advanced cancer, as opposed to 36% of non–Native Americans.




Source. 2000 US Census data.

**FIGURE 1—Native American population, by county, for the region using Rapid City Regional Hospital.**

## KEY FINDINGS

- Native Americans in the IHS Northern Plains Region have significantly higher age adjusted rates of cancer mortality than the general US population.
- Native Americans in western South Dakota present with more advanced stages of cancer than do non-Native Americans in the region.
- The Cancer Disparities Research Partnership project at Rapid City Regional Hospital is positioned to have a significant impact on cancer health care for the Native American population of western South Dakota.
- Building trust requires going through the full tribal and IHS approval process, including taking time to educate and to answer questions.
- Building trust also requires a commitment to providing needed services in the community over time and in a culturally appropriate way.

A sizeable literature exists on the barriers to equal health-related behaviors, health care, and outcomes for minority groups in the United States. In a review of the published literature through 1996, Guidry et al. identified barriers such as communication problems between patients and providers, lack of information on side effects, cost of treatment, difficulty in obtaining and maintaining insurance coverage, and absence of social support networks.[8] Other studies on the barriers to healthy behavioral choices and to timely and effective health care for minority groups have identified additional problems, including the need for social support[9]; poor communication between patient and provider regarding different understandings of health and disease[10]; fear, language barriers, and lack of education and acculturation[11]; perceived racial, economic, and gender bias[12]; lack of a regular doctor[13]; lack of cultural competence on the part of nurses[14]; and low levels of health literacy.[15]

Barriers to timely and effective cancer diagnosis and treatment for Native Americans in western South Dakota include the following: lack of knowledge of the disease, its screening, and treatment; logistical problems (e.g., transportation, finances, family care, communications) in accessing cancer-related health care; lack of trust, hope, or emotional support concerning cancer treatment and recovery; and the red tape involved in dealing with multiple health care entities, which leads to problems in obtaining test results, understanding options, being referred, and making payments. Taken together, these data and barriers indicate that in our region, cancer outcomes for Native Americans are significantly poorer than those for non–Native Americans. We have outlined obstacles to and solutions for a cancer research program attempting to resolve these disparities.

## THE PROJECT

The National Cancer Institute's Cancer Disparities Research Partnership (CDRP) program has funded 6 sites across the United States to research and reduce cancer mortality disparities among various minority populations. Rapid City Regional Hospital's CDRP grant, "Enhancing Native American Participation in Radiation Therapy Trials," provides clinical trials, behavioral research, a genetic protocol, Patient Navigator services, travel assistance, and community education for members of 3 Lakota tribes in western South Dakota (Oglala, Rosebud, and Cheyenne River Sioux tribes) as well as the Native American population in Rapid City. The 5-year project was initiated in late 2002.

The goal of this grant project is to reduce cancer mortality rates for Native Americans in the region. Specific objectives that support this goal are as follows:

1. To document the major factors responsible for cancer health disparities in the Native American population served by Rapid City Regional Hospital;

**TABLE 1—Percentages of Native Americans and Non-Native Americans Presenting at Rapid City Regional Hospital With Advanced (Stage III or IV) Cancer: Tumor Registry Data, 1990–2000**

| Cancer Type | % Presenting With Stage III or IV Disease (n) | |
|---|---|---|
| | Native American | Non-Native American |
| Lung | 72 (92/127) | 68 (669/989) |
| Breast | 16 (12/75) | 10 (111/1127) |
| Colorectal | 48 (26/54) | 40 (298/739) |
| Prostate | 44 (22/50) | 30 (281/945) |
| Cervix | 53 (8/15) | 26 (13/51) |

2. To determine whether shorter, but equally effective, courses of treatment will enhance the acceptability and completion rate of radiotherapy; and

3. To ascertain whether there may be a genetic basis for anecdotal reports that Native Americans experience increased radiation toxicities.

With a staff of 10 (including 4 field staff members), 2 collaborating partner institutions (University of Wisconsin–Madison and Mayo Clinic–Rochester), numerous consultants, and 8 unique research protocols, this large project is positioned to significantly affect the approach to cancer-related health care for the Lakota Nation.

## OBSTACLES TO OVERCOME

The project has had to overcome a number of serious obstacles. The most immediate of these has been the need to obtain multiple approvals for each research protocol. Clinical protocols (brachytherapy and tomotherapy radiation treatment trials) have each required the approval of 4 institutional review boards (IRBs): those of the University of Wisconsin, the Rapid City Regional Hospital, the Aberdeen Area IHS, and the national IHS. Survey and Patient Navigator protocols have each required the approval of 3 IRBs: those of the Rapid City Regional Hospital, the Aberdeen Area IHS, and the national IHS. The genetic protocol will require approval from 5 IRBs (those already named and the Mayo Clinic IRB). Furthermore, before considering these protocols, the IRB of the Aberdeen Area IHS requires resolutions of support from each of the 3 tribes involved, from the chief executive officers at the IHS hospitals on the 3 reservations and in Rapid City, and from the 18-member Aberdeen Area Tribal Chairmen's Health Board. Arguably, IHS IRB approvals were not required because we are not using any IHS staff, data, or patient records for the research; however, we felt these approvals were extremely important for maintaining good relationships and ensuring broad-based community support for our project.

The project's approach to this daunting task has been one of patience, persistence, and relationship building. Staff members meet frequently with and make periodic presentations to tribal councils, tribal health boards, IHS decisionmakers, community leaders, and the Aberdeen Area Tribal Chairmen's Health Board. Working groups were held on each reservation and in Rapid City to receive input on survey questions. A biweekly to monthly radio show allowed us to talk about the importance of early detection and treatment for cancer. A community genetics education curriculum was being developed to explain the value of the cancer-related genetic test we plan to offer.

We have received approval for all of our 8 protocols from all the relevant IRBs and tribal entities and from the Aberdeen Area Tribal Chairmen's Health Board. We gained the necessary support from the tribes by explaining our research protocols, making the case for their usefulness to individual cancer patients and to the tribe, answering questions openly, and providing frequent updates to decisionmakers and community members. For each tribal resolution of support, we included language ensuring that research results would be shared with the tribe. We could not, however, share raw data, because this would constitute a violation of confidentiality. Therefore, we shared results in the form of quarterly written updates that were presented to tribal councils and the Tribal Chairmen's Health Board each time we met with them. Moreover, our IHS IRB approvals required us to obtain tribal approvals prior to publications about the study. For example, we have letters of approval from all 3 tribes for this article.

Another set of obstacles reflects the barriers that face Native Americans needing cancer treatment: long travel distances; lack of screening opportunities; lack of education about cancer; IHS funding shortfalls; the red tape of patient referrals; payment problems involving the IHS, Medicare, Medicaid, the Department of Veterans Affairs, TRICARE (military health care program), and private insurers; and the refusal of some payers to cover clinical trials. Our staff mitigate these issues on a daily basis, with help from the tribes, the IHS, contract health offices, the National Breast and Cervical Cancer Early Detection Program, nonprofit organizations such as the American Cancer Society, and others. Constant networking, communication, and brainstorming allow us to keep everyone in the loop and look for creative ways to resolve the various roadblocks that arise. The long travel distances from the reservation to the Cancer Care Institute are mitigated through a grant fund that provides money to cancer patients for gas, food, and lodging as part of our Patient Navigator program. Sophisticated telemedicine equipment provided by the grant will be used for patient consultations so that lengthy trips can be avoided where possible.

A third obstacle is the fact that Rapid City Regional Hospital had not received federal research grants in the past and thus had not developed either the necessary infrastructure for administering grants or a culture that supports research. Other hospitals located close to rural Native American populations are likely to face the same situation. The National Cancer Institute's CDRP program anticipated this problem, providing for advice and mentoring from 2 experienced partner institutions (University of Wisconsin–Madison and Mayo Clinic–Rochester).

Perhaps the most fundamental obstacle to this project is the historical reality of relationships

between the Lakota and non–Native American (White) populations over the past century and a half. Many Native Americans in western South Dakota have had experiences or heard stories that led them to doubt their welcome in Rapid City and at Rapid City Regional Hospital. Some Native Americans, particularly those with a more traditional perspective, do not trust Western medicine, medical research, or genetic testing.

Building trust, therefore, is an essential component of all our activities. Trust requires openness, honesty, culturally appropriate messages, culturally integrated staff, services that people can appreciate, long-term commitments, consistency, patience, and time. As Lakota people often say, "We have to do this in a good way." Without such trust, none of our research studies would receive the approvals required to begin, let alone the necessary community cooperation required to succeed. If the project succeeds in developing effective programs that help lower cancer mortality rates, it will constitute a milestone in reconciliation as well as in the interrelated challenge of reducing health care disparities. ■

## About the Authors

*Deborah Rogers is with Rapid City Regional Hospital, Rapid City, SD. Daniel G. Petereit is with the John T. Vucurevich Cancer Care Institute, Rapid City Regional Hospital, Rapid City, SD, and the Department of Human Oncology, University of Wisconsin, Madison.*

*Requests for reprints should be sent to Daniel G. Petereit, MD, John T. Vucurevich Cancer Care Institute, 353 Fairmont Blvd, Rapid City, SD 57701 (e-mail: dpetereit@rcrh.org).*

*This report was accepted April 15, 2005.*

*doi:10.2105/AJPH.2004.053645*

Note. *The opinions expressed in this report are those of the authors and do not necessarily reflect the views of the Indian Health Service.*

## Contributors

D. Rogers wrote and edited the report and manages the implementation of nonclinical aspects of the research. D.G. Petereit conceived of the idea for the research and directs the research and contributed ideas for and revised the report.

## Acknowledgments

We gratefully acknowledge the funding and support of the Cancer Disparities Research Partnership, Radiation Research Program, National Cancer Institute (contract N01-CO-12400). Administrative support and facilities are provided by Rapid City Regional Hospital.

We thank the key individuals who have been instrumental in designing the research described in this report: Minesh Mehta of the University of Wisconsin Comprehensive Cancer Center; Norm Coleman, Frank Govern, and Rosemary Wong of the Radiation Research Program, National Cancer Institute; Christen Osburn of SAIC-Frederick, Inc (government contractor); Judith Kaur of Mayo Clinic–Rochester Comprehensive Cancer Center; Linda Burhansstipanov of Native American Cancer Research Corporation; and Ivan Helbig of Rapid City Regional Hospital. We especially appreciate the efforts of our staff who are on the front lines daily, building community trust and confronting cancer: Kevin Molloy, Cathey Duchenaux, and Mary Reiner, Rapid City; Caroline Spotted Tail, Rosebud; Raylene LeBeau, Cheyenne River; and Scotty Crawford, Pine Ridge.

## Human Participant Protection

All 8 study protocols received resolutions of support from the Oglala Sioux Tribal Council, the Rosebud Sioux Tribal Council, the Cheyenne River Sioux Tribal Council, the Pine Ridge Indian Hospital CEO, the Cheyenne River Public Health Service Hospital CEO, the Rosebud IHS Hospital CEO, the Rapid City Public Health Service Indian Hospital CEO, and the Aberdeen Area Tribal Chairmen's Health Board, and have received approvals as required from institutional review boards at the University of Wisconsin, Rapid City Regional Hospital, Aberdeen Area IHS, and National IHS. The retrospective chart review referred to in Table 1 was approved by Rapid City Regional Hospital's institutional review board.

## References

1. Bobinski MA. Health disparities and the law: wrongs in search of a right. *Am J Law Med.* 2003;29:363–380.

2. Geiger HJ. Racial and ethnic disparities in diagnosis and treatment: a review of the evidence and a consideration of causes. In: Smedley BD, Smith AY, Nelson AR, eds. *Unequal Treatment: Confronting Racial and Ethnic Disparities in Health Care.* Washington, DC: Institute of Medicine, National Academies Press; 2003:415–454.

3. Sateren WB, Trimble EL, Abrams J, et al. How sociodemographics, presence of oncology specialists, and hospital cancer programs affect accrual to cancer treatment trials. *J Clin Oncol.* 2002;20:2109–2117.

4. Hayes MA, Smedley BD. *The Unequal Burden of Cancer: An Assessment of NIH Research and Programs for Ethnic Minorities and the Medically Underserved.* Washington, DC: Institute of Medicine, National Academies Press; 1999.

5. Espey DK, Paisano RE, Cobb N. *Cancer Mortality Among American Indians and Alaska Natives: Regional Differences, 1994–1998.* Rockville, Md: Indian Health Service; 2003. IHS publication 97-615-28.

6. *2000–2001 Regional Differences in Indian Health.* Rockville, Md: Indian Health Service, US Dept of Health and Human Services; 2002.

7. Petereit DG, Rogers D, Helbig P, et al. Geographic distance from the cancer center may be a treatment barrier for American Indians undergoing radiotherapy. Paper presented at: Intercultural Cancer Council 9th Biennial Symposium on Minorities, the Medically Underserved and Cancer; March 24–28, 2004; Washington, DC.

8. Guidry JJ, Gressinger A, Aday LA, Winn RJ, Vernon S, Throckmorton TA. Barriers to cancer treatment: a review of published research. *Oncol Nurs Forum.* 1996;23:1393–1398.

9. Cook GC, Wilson ME. Social support and cancer screening in African American, Hispanic, and Native American women. *Cancer Pract.* 1998;6:31–37.

10. Ashton CM, Haidet P, Paterniti DA, et al. Racial and ethnic disparities in the use of health services: bias, preferences, or poor communication? *J Gen Intern Med.* 2003;18:146–152.

11. Otero-Sabogal R, Owens D, Canchola J, et al. Mammography rescreening among women of diverse ethnicities: patient, provider, and health care system factors. *J Health Care Poor Underserved.* 2004;15:390–412.

12. La Veist TA, Nickerson KJ, Bowie JV. Attitudes about racism, medical mistrust, and satisfaction with care among African American and white cardiac patients. *Med Care Res Rev.* 2000;57:146–161.

13. Cornelius LJ, Smith PL, Simpson GM. What factors hinder women of color from obtaining preventive health care? *Am J Public Health.* 2002;92:535–539.

14. Gonzalez RI, Gooden MR, Porter CP. Eliminating racial and ethnic disparities in health care. *Am J Nurs.* 2000;100:56–58.

15. Lindau ST, Tomori C, Lyons T, Langseth L, Bennett CL, Garcia P. The association of health literacy with cervical cancer prevention knowledge and health behaviors in a multiethnic cohort of women. *Am J Obstet Gynecol.* 2002;186:938–943.

Mutagenesis Advance Access published September 29, 2005
Mutagenesis doi:10.1093/mutage/gei056

# Uranyl acetate induces *hprt* mutations and uranium–DNA adducts in Chinese hamster ovary EM9 cells

**Diane M.Stearns*, Monica Yazzie, Andrew S.Bradley, Virginia H.Coryell, Jake T.Shelley, Adam Ashby, Craig S.Asplund and R.Clark Lantz[1]**

Department of Chemistry and Biochemistry, Northern Arizona University, PO Box 5698, Flagstaff, AZ 86011-5698 and [1]Department of Cell Biology and Anatomy, University of Arizona, Tucson, AZ, USA

**Questions about possible adverse health effects from exposures to uranium have arisen as a result of uranium mining, residual mine tailings and use of depleted uranium in the military. The purpose of the current study was to measure the toxicity of depleted uranium as uranyl acetate (UA) in mammalian cells. The activity of UA in the parental CHO AA8 line was compared with that in the XRCC1-deficient CHO EM9 line. Cytotoxicity was measured by clonogenic survival. A dose of 200 μM UA over 24 h produced 3.1-fold greater cell death in the CHO EM9 than the CHO AA8 line, and a dose of 300 μM was 1.7-fold more cytotoxic. Mutagenicity at the hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) locus was measured by selection with 6-thioguanine. A dose of 200 μM UA produced ~5-fold higher averaged induced mutant frequency in the CHO EM9 line relative to the CHO AA8 line. The generation of DNA strand breaks was measured by the alkaline comet assay at 40 min and 24 h exposures. DNA strand breaks were detected in both lines; however a dose response may have been masked by U–DNA adducts or crosslinks. Uranium–DNA adducts were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) at 24 and 48 h exposures. A maximum adduct level of 8 U atoms/$10^3$ DNA-P for the 300 μM dose was found in the EM9 line after 48 h. This is the first report of the formation of uranium–DNA adducts and mutations in mammalian cells after direct exposure to a depleted uranium compound. Data suggest that uranium could be chemically genotoxic and mutagenic through the formation of strand breaks and covalent U–DNA adducts. Thus the health risks for uranium exposure could go beyond those for radiation exposure.**

## Introduction

Questions about possible adverse health effects from environmental and occupational exposures to uranium have arisen as a result of uranium mining, residual mine tailings, and the use of depleted uranium in the military. Depleted uranium is uranium that has higher levels of $^{238}$U and lower levels of $^{235}$U and $^{234}$U relative to natural uranium. Over half of the US uranium reserves are believed to exist in the Four Corners area of the Southwestern United States (1). It is estimated that over 300 tons of depleted uranium were used during Gulf War I (2), but estimates for Gulf War II have not yet been reported. The impact of these high levels of environmental uranium on exposed populations is of growing concern.

The adverse health effects from occupational and experimental uranium exposures that have been established most significantly include lung cancer, from exposure to $^{222}$radon that is produced through the radioactive decay of $^{238}$U (3,4), and chemically induced kidney toxicity (5); however, bladder damage (6), birth defects (7) and chromosomal aberrations (8) have also been reported. Thorough epidemiological data for health effects from either environmental exposures to uranium tailings or military exposures to depleted uranium are currently lacking due to insufficient study of both Native American populations other than miners, and the short time span since initial military exposures to DU weapons and munitions. However, evaluation of DU-exposed veterans is in progress (9).

Previous work has shown that depleted uranium as uranyl acetate (UA) produced DNA strand breaks in the presence of vitamin C, which suggested a chemical rather than radiological mechanism (10). The purpose of the current study was to measure the potential for depleted uranium as UA to be toxic in mammalian cells. Depleted uranium was used because, besides being the commercially available form of uranium, it provides less likelihood for chemical effects to be masked by radioactivity. A soluble form of U(VI) was tested here because of an interest in environmental exposure through drinking water; however, it is not assumed that insoluble uranium provides no risk environmentally. Based on previous work *in vitro* (10) it was expected that DNA strand breaks may be formed in cultured cells, and it was presumed that if strand breaks were occurring then those lesions could be relatively easily repaired. For this reason experiments were carried out in both the parental CHO AA8 line and the CHO EM9 line, which has reduced levels of the XRCC1-DNA ligase III complex (11), and is therefore sensitive to DNA strand breaks (12). It was hypothesized that if UA caused direct DNA damage in CHO cells then it should be more cytotoxic and mutagenic in the repair-deficient EM9 line than the parental AA8 line. Results supported these hypotheses; however, the DNA damage produced in CHO cells was not limited to strand breaks, but included U–DNA adducts.

## Materials and methods

### *Reagents and chemicals*

Depleted uranium as uranyl acetate dihydrate [6159-44-0] (UA), with a $U^{234}/U^{238}$ activity ratio of 0.12 (10), was obtained from Spectrum Chemical Mfg Corp. (Gardena, CA). 2-Amino-6-mercaptopurine (6-thioguanine) [154-42-7] (Sigma Chemical Co., St Louis, MO) was used as received.

### *General cell culture conditions*

Chinese hamster ovary AA8 and EM9 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells at passage 3 were thawed from cryopreservation, cultured in αMEM (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), antibiotic/antimycotic

*To whom correspondence and reprint requests should be addressed. Tel: +1 928 523 4460; Fax: +1 928 523 8111; Email: Diane.Stearns@nau.edu

© The Author 2005. Published by Oxford University Press on behalf of the UK Environmental Mutagen Society.
All rights reserved. For permissions, please email: journals.permissions@oxfordjournals.org

(100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B) (Sigma) and 1 mM glutamine (Gibco-BRL, Rockville, MD). Cells were maintained at 37°C in a 5% $CO_2$/air humidified incubator calibrated with a Fyrite analyzer (Bacharach Co., Pittsburgh, PA).

*Cytotoxicity measurements*

Cytotoxicity, as decreased cell survival, was determined by measuring colony-forming ability in the CHO AA8 and CHO EM9 lines. Cytotoxicity measurements were carried out after incubation with UA for 24 h. Cells were seeded at $8 \times 10^5$ cells per 100 mm plate, allowed to adhere for over ~20 h, and treated with sterile-filtered aqueous solutions of UA (0–300 μM) for 24 h. Upon completion of exposure, cells were trypsinized, quantified on a Z1 Coulter Particle Counter (Beckman Coulter, Inc., Miami, FL) and reseeded at 200 cells (AA8) or 300 cells (EM9) per 60 mm dish in quadruplicate. After 7 days all dishes were stained with crystal violet and the colonies were counted. Cell survival was calculated as percent colonies in treated dishes relative to untreated controls. Plating efficiency was 86% for the AA8 line and 56% for the EM9 line.

*Mutagenicity measurements*

The HPRT assay was carried out following published procedures (13) with minor modifications. Cells were exposed to UA at 100–300 μM for 24 h as described above. Cells were harvested and analyzed for cell survival with a 7 day colony formation assay as described above. From the same cell populations harvested for cell survival measurements after 24 h treatment times, approximately $1 \times 10^6$ cells were reseeded in 100 mm dishes and that amount was passaged every 3 days until day 9 post-treatment. This expression time was found in previous studies (14) to be optimum (data not shown), and is consistent with recommendations for this assay (13). After this total 10-day expression time cells were seeded again for colony-forming ability as described above, and $2.5 \times 10^5$ cells were seeded in quadruplicate in 100 mm dishes and incubated in medium containing 11 μg/ml of 6-thioguanine (6-TG) for 7–8 days for mutant selection. Data are expressed as mutants per $10^6$ surviving cells, calculated from the observed 6-TG-resistant colonies and the 10-day clonogenic values. Average induced mutant frequency and average mutant increase above background were calculated from the differences and ratios of individual experiments. Experiments were repeated 4–7 times.

*Single cell gel electrophoresis (comet assay)*

The ability of UA to produce DNA strand breaks in CHO AA8 and CHO EM9 cells was measured by the alkaline comet assay following recommended procedures (15,16). Briefly, CHO AA8 or CHO EM9 cells were seeded at $8 \times 10^5$ cells/ml and exposed to UA after cell attachment. Cells were treated with sterile aqueous solutions of 50–300 μM UA for 40 min or for 24 h at 37°C. As a positive control for strand breaks and oxidative damage, cells were treated with 40 or 80 μM $H_2O_2$ for 40 min at 37°C. Untreated cells served as a negative control. Cells were harvested by scraping in dim light, pelleted and placed on ice. Microgels were prepared in duplicate on MGE slides (Erie Scientific Inc., Portsmouth, NH) in four layers as recommended (16). All slides were subjected to lysis solution (2.5 M NaCl, 100 mM EDTA tetrasodium salt, 10 mM Tris, pH 10, 1% sodium lauroyl sarcosine, 1% Triton X-100) for 2 h at 4°C.

One set of duplicate slides for each dose was incubated with *Escherichia coli* formamidopyrimidine-DNA glycosylase (FPG) using the FLARE Assay kit (Trevigen, Inc., Gaithersburg, MD) after the 2 h lysis step, in order to detect the presence of oxidative damage. Slides were rinsed in 1× FLARE buffer for 15 min, and the FPG enzyme, at 50 U in 200 μl, was added to the slides for 30 min at 37°C. These slides were then combined with other duplicate slides for the remainder of the assay.

All slides were then subjected to unwinding in alkaline buffer (300 mM NaOH, 1 mM EDTA, 0.2% DMSO, 0.1% 8-hydroxyquinoline, pH >13) for 20 min, followed by electrophoresis for 15 min on a horizontal electrophoresis unit (MGE, Techniport, Inc.) at 250 mA and 4°C with buffer recirculation of 100 ml/min.

After electrophoresis, slides were neutralized in 1 M ammonium acetate in ethanol for 15 min at ambient temperature, followed by incubation in 1 mg/ml spermine in 66% ethanol for 15 min at ambient temperature. Slides were air-dried in the dark before staining. Slides were prestained for 1 min with a 60 μl volume of 5% sucrose and 1 mM monosodium phosphate. Slides were stained 30 min prior to analysis with 200 μl of a 1:10 000 dilution of SYBR® Green (Molecular Probes, Eugene, OR) followed by 50 μl of Vectashield (Vector laboratories, Inc., Burlingame, CA).

Slides were analyzed for DNA damage on an Olympus BX51 epifluorescence microscope equipped with an LAI Comet Assay Analysis System (Loats Associates, Inc., Westminster, MD) at 40× magnification. Tail moment (tail length × percentage of DNA in tail) was measured in 50 cells for each treatment, positive and negative controls, and independent experiments were repeated in triplicate or quadruplicate. The average of 50 cells was calculated for each treatment, and reported data represent the mean ± SEM of the individual averages of the 3–4 independent experiments.

*Measurement of uranium/DNA-P binding by ICP-OES*

CHO AA8 or CHO EM9 cells were seeded in duplicate 100 mm dishes at $5 \times 10^5$ or $7 \times 10^5$ cells per plate, respectively, and were either allowed to grow for 48 h and treated with UA at final concentrations of 0–300 μM for 24 h or were allowed to grow for 24 h and treated with 0–300 μM UA for 48 h. At 5 min before harvesting one set of duplicate dishes was treated with 0–300 μM UA to serve as a 'zero time point control' to measure background or membrane-bound uranium that could be carried along in harvested cells to artificially interact with DNA during DNA extraction. Values of U–DNA for zero time points were not statistically different from untreated cells (data not shown). Cells were washed three times with PBS, harvested with trypsin, and cell suspensions from duplicate treatments were combined, pelleted and stored at −4°C until DNA was extracted. Cells were subsequently thawed and lysed in 10 mM Tris, 5 mM EDTA, 5% SDS, 0.2 M NaCl. RNA was removed by incubating samples in lysis buffer containing 2 U of pancreatic RNase A (Sigma) at 37°C for 30 min. DNA was extracted with 25:24:1 phenol:$CHCl_3$:isoamyl alcohol, separated by centrifugation in Light Phase Lock Gel™ tubes, twice precipitated with isopropyl alcohol, washed with 75% and 100% ethanol, air-dried, and digested in 200 μl of 20% $HNO_3$ and 50 μl of 30% $H_2O_2$ by heating for 1 h at 80°C. Samples were then diluted to a final volume of 3 ml containing 0.1 p.p.m. ytterium as an internal standard. The digested samples were then assayed for uranium and phosphorous on a PerkinElmer Optima 4300DV inductively coupled plasma optical emission spectrometer (ICP-OES) with a meinhard nebulizer and a cyclonic spray chamber. The plasma operated at 1300 W with a sample introduction rate of 1.50 ml/min. The plasma, auxiliary, and nebulizer flow rates were 15, 0.2, and 0.80 l/min, respectively. The emission wavelengths used for uranium and phosphorous were 385.958 and 213.617 nm, respectively. The metal-nucleotide binding ratios were calculated as the moles of uranium per mole of phosphorous in the sample. The limits of detection were 1.805 p.p.b. ($7.58 \times 10^{-9}$ M) for uranium, and 9.322 p.p.b. ($3.01 \times 10^{-7}$ M) for phosphorous. The limits of quantitation were 6.402 ppb ($2.69 \times 10^{-8}$ M) for uranium, and 20.71 ppb ($6.69 \times 10^{-7}$ M) for phosphorous.

*Statistics*

Statistical significance was evaluated by ANOVA using the Tukey *post hoc* test. Differences were considered significant at $P < 0.05$. Statistical outliers were verified by Grubbs' test (extreme studentized deviate method), $P < 0.05$.

## Results

It was initially hypothesized that if UA caused DNA strand breaks *in vitro* (10) then UA should be more toxic in the strand break-sensitive CHO EM9 line than in the parental CHO AA8 line. This hypothesis was tested by measuring the cytotoxicity and mutagenicity of UA in CHO AA8 and EM9 cells.

The cytotoxicity of UA was measured by a colony formation assay. Cell survival of UA decreased with increasing doses in both CHO AA8 and CHO EM9 cells after 24 h exposures (Figure 1). UA was more cytotoxic in the EM9 line than in

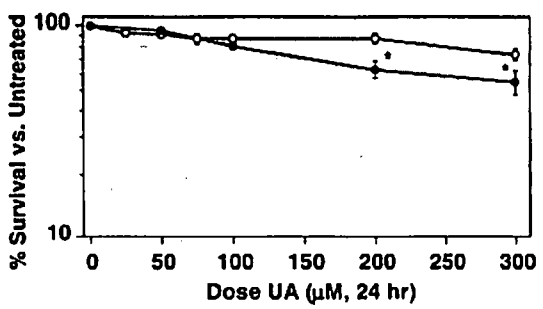



Fig. 1. Cytotoxicity of UA in CHO-AA8 (open circles) and CHO-EM9 (closed circles) cells after 24 h exposures. Cells were treated and assayed for 7–8 day colony formation as described in the text. Data represent mean ± SEM for $n$ = 8–11 independent experiments. The asterisk indicates statistical significance between equivalent doses in the AA8 and EM9 lines at $P < 0.05$.

Table I. Cell survival and mutation induction in CHO AA8 and CHO EM9 cells treated with UA for 24 h

| Treatment (24 h) | Survival[a] (10 Days) | | Average mutants per $10^6$ survivors | | AIMF[b] | | Average mutant increase above background[c] | |
|---|---|---|---|---|---|---|---|---|
| | AA8 | EM9 | AA8 | EM9 | AA8 | EM9 | AA8 | EM9 |
| Untreated | 100 ± 0 | 100 ± 0 | 5.1 | 5.3 | (0) | (0) | (1.0) | (1.0) |
| 100 μM UA | 99 ± 3.2 | 85 ± 4.8 | 9.8 | 16.2 | 4.5 | 11.0 | 5.7 | 4.8 |
| 200 μM UA | 98 ± 3.1 | 90 ± 1.9 | 11.8 | 36.6 | 6.6 | 31.3[d] | 4.0 | 7.8 |
| 300 μM UA | 102 ± 3.0 | 85 ± 2.6 | 10.7 | 27.2 | 5.9 | 22.0 | 2.3 | 4.7 |

[a]Colony formation measured after 24 h treatments plus 9 day growth time for expression of mutant phenotype. Colony formation after 24 h exposures is shown in Figure 1.
[b]Treatment MF–control MF per $10^6$ viable cells averaged from differences in individual experiments.
[c]Treatment MF/control MF averaged from ratios in individual experiments.
[d]Significantly different from equivalent dose in AA8 cells, $P < 0.05$.

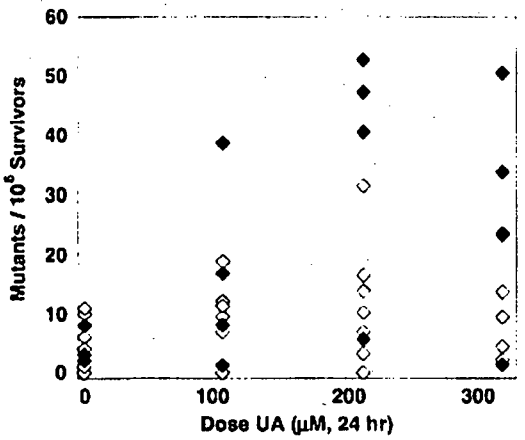



Fig. 2. 6-Thioguanine-resistant cells obtained after 24 h exposure of CHO-AA8 (open diamonds) and CHO-EM9 (closed diamonds) cells to UA. Methods are described in the text. Each data point refers to an individual experiment.

the AA8 line, with the 200 μM dose producing 12 and 37% cell death in the AA8 and EM9 lines, respectively, and the 300 μM dose producing 26 and 45% cell death in the AA8 and EM9 lines, respectively. The observation that UA was more cytotoxic in the repair-deficient EM9 line than the parental AA8 line supported the initial hypothesis and was interpreted to suggest that UA caused DNA damage in CHO cells.

The mutagenicity of UA was measured at the *hprt* locus by a selection of cells resistant to 6-thioguanine (6-TG). After 24 h treatment and a 9 day recovery time to allow for expression of the mutant phenotype, survival of UA-treated cells recovered to ≥98% in the AA8 line and ≥85% in the EM9 line (Table I). UA was weakly mutagenic in the two CHO lines (Figure 2). The untreated AA8 cells averaged 5.1 spontaneous mutations (range 0–11), and the EM9 line averaged 5.3 spontaneous mutations (range 2–8) (Table I). The highest induced mutant frequency was observed in the EM9 line for the 200 μM and 300 μM doses, at 31.3 and 22, respectively, which were ~5-fold and ~4-fold higher than the frequency observed for equivalent doses in the AA8 line. These data were consistent with the interpretation that if UA caused direct DNA damage in CHO cells, then at least some, but perhaps not all, of the resulting DNA lesions were mutagenic.

It was hypothesized that if UA was mutagenic in repair-deficient cells then DNA damage should be occurring either by direct uranium–DNA interactions or by generation of reactive oxygen species. This hypothesis was tested by measuring DNA damage as strand breaks, oxidative damage and uranium–DNA adducts in CHO EM9 and AA8 cells exposed to UA.

The presence of DNA strand breaks was measured by the alkaline comet assay in cells exposed to 50–300 μM UA for 40 min and 24 h. Exposure to 40 and 80 μM $H_2O_2$ for 40 min at 37°C served as the positive control. Hydrogen peroxide showed an increase in tail moment with increasing dose in both cell lines (Figure 3A and B). In the AA8 line post-treatment with FPG significantly increased the tail moment for both $H_2O_2$ doses ($P \leq 0.01$), suggesting that oxidative damage was present, as expected (Figure 3A). However, FPG did not produce a significant increase in $H_2O_2$-induced tail moment in the EM9 line (Figure 3B). The mean tail moments for CHO EM9 cells exposed to 40 or 80 μM $H_2O_2$ were slightly lower than those in the AA8 line. The mean tail moments for CHO EM9 cells exposed to $H_2O_2$ with FPG post-treatment were significantly lower than equivalent exposures in the AA8 line for both 40 and 80 μM doses ($P < 0.02$). These results are consistent with a study that found less $H_2O_2$-induced DNA strand breaks in the EM9 line relative to the AA8 line (17).

Exposure to UA at 40 min resulted in significant increases in tail moments relative to untreated controls for all doses (50–300 μM) in both cell lines ($P < 0.05$); however, no dose response was apparent in either cell line (Figure 3A and B). There was no significant difference in tail moments between lines for equivalent UA doses, nor did FPG have a significant effect on increasing tail moment for any dose of UA in either line.

Exposure to UA at 24 h in the AA8 and EM9 lines produced similar increases in tail moment relative to untreated controls and a similar lack of clear dose response in both lines (Figure 3C and D). Treatment with UA plus post-treatment exposure to FPG yielded no significant increase in tail moments relative to treatments with UA alone in either cell line. These results for both 40 min and 24 h exposures could mean that UA did not produce any formamidopyrimidines, and could thus be interpreted to suggest that oxidative damage did not occur in UA-treated cells under these conditions. Data could also be interpreted to suggest that DNA strand breaks did occur in UA-exposed cells; however, the dose response in the comet assay could have been masked by the presence of another lesion, for example DNA crosslinks (*vide infra*).

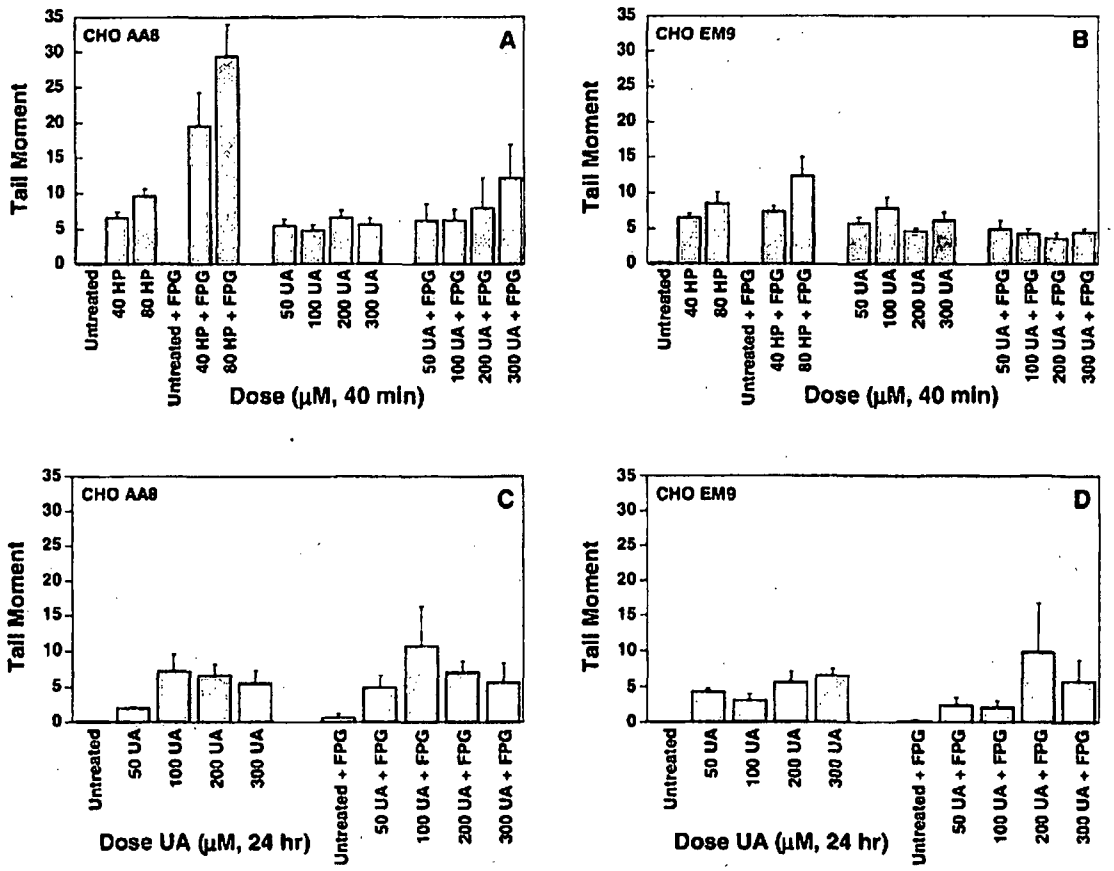



Fig. 3. Analysis of DNA damage induced by UA and $H_2O_2$ by the comet assay. (A) CHO AA8 cells exposed to $H_2O_2$ or UA for 40 min at 37°C. (B) CHO EM9 cells exposed to $H_2O_2$ or UA for 40 min at 37°C. (C) CHO AA8 cells exposed to UA for 24 h. (D) CHO EM9 cells exposed to UA for 24 h. Cells were analyzed for strand breaks, or were post-treated with FPG and analyzed for oxidative damage. Data represent mean tail moment ± SEM for $n$ = 3–4 independent experiments. Within each experiment 50 cells were scored for each dose.

Uranium is a metal that forms bonds with biological molecules; thus general uranium-DNA adducts could represent another potential class of DNA lesions. The ability of UA to produce uranium–DNA adducts was therefore measured by ICP-OES in both cell lines. Cells were exposed to 0–300 μM UA for 24 and 48 h. DNA was extracted and precipitated from washed, harvested cells. After digestion in $HNO_3/H_2O_2$, concentrations of uranium and phosphorous were measured by ICP-OES and ratios of uranium to DNA-P were calculated. Experiments were carried out with and without RNaseA to compare uranium binding to DNA versus total nucleic acid. Data showed that uranium–DNA adducts existed on the order of a few U atoms per thousand nucleotides, and increased with increasing dose and increasing exposure time for 24 and 48 h treatments in both cell lines (Figure 4).

The effect of RNase A on adduct levels was only significant in the AA8 line at the highest dose tested. In the AA8 line, at the 24 h exposure there were 2.4-fold more ($P < 0.0001$) U–DNA adducts in samples exposed to RNase A relative to samples for which RNA was not degraded (Figure 4). At the 48 h exposure there were 2.5-fold more ($P < 0.001$) U–DNA adducts in RNase-treated samples. In the EM9 line there was no difference between uranium adducts in DNA versus total nucleic acid.

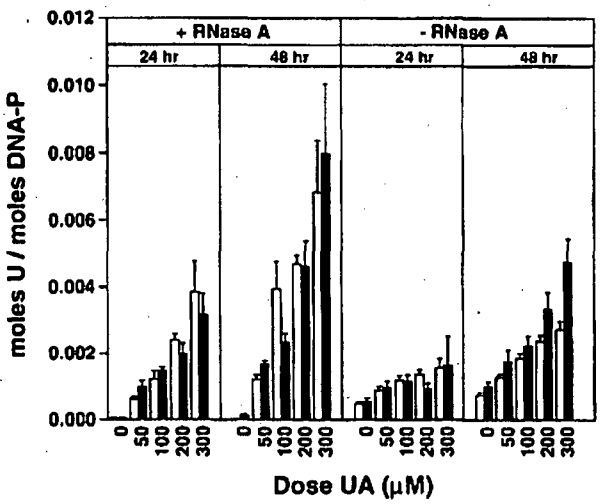



Fig. 4. Measurement of uranium–DNA binding in CHO AA8 (open bars) versus CHO EM9 (closed bars) cells exposed to UA for 24 or 48 h by ICP-OES. Uranium was found bonded to DNA after incubation with 2U RNase A (+RNase A) and without (−RNase A). Data represent mean ± SEM for $n$ = 4–9 experiments.

In general, there was no significant difference in U–DNA adducts between the AA8 and EM9 lines at either exposure times. In samples not exposed to RNase A, the EM9 line showed 1.7-fold higher U-nucleic acid adducts than the AA8 line after 48 h; however, this difference was not seen after the 24 h exposure, nor was it evident in samples exposed to RNase A.

## Discussion

Measurements of UA-induced DNA toxicity were carried out in both the repair-proficient CHO AA8 line and the repair-deficient CHO EM9 line. The compromised DNA repair in the EM9 line is due to expression of 4-fold lower DNA ligase IIIα and ~10-fold lower X-ray repair cross-complementing gene I protein (XRCC1) relative to the parental line (11). Ligase IIIα catalyzes the rejoining of the DNA phosphodiester backbone and is thus involved in base excision repair and repair of DNA strand breaks (18). The XRCC1 protein has no catalytic activity but forms complexes with ligase IIIα, as well as with poly(ADP-ribose) polymerase 1 (PARP-1), PARP-2 and several other DNA repair proteins (19). The observation that UA was more cytotoxic in the CHO EM9 line than the CHO AA8 line, coupled with the assumption that the only difference between the two lines was their ability to repair DNA damage, was inferred to suggest that UA caused direct DNA damage that was differentially repaired in these two lines.

The lack of a significant difference between the two cell lines in terms of DNA strand break production was unexpected; however, strand breaks were estimated by mean tail moment in the comet assay, and the presence of other forms of damage may have interfered in the migration of DNA in that assay. For example, if U–DNA adducts existed in the form of crosslinks, or if crosslinks that did not contain uranium were present, those lesions could decrease tail migration, as has been observed for platinum and other crosslinking agents (20).

Comparisons can also be made between the two lines in terms of cytotoxicity. The 2- to 3-fold increased cytotoxicity of UA in the EM9 versus AA8 lines places UA in the same category as chemicals known to cause DNA strand breaks and DNA crosslinks, for example $H_2O_2$ (17), chromate and mercuric chloride (21), UVA light (22) and near visible and blue light (23). The observation that monofunctional alkylating agents ethyl methanesulfonate and methyl methanesulfonate were >10-fold more cytotoxic in the EM9 versus AA8 lines (24) is consistent with the interpretation that UA may not form monofunctional adducts or apurinic/apyrimidinic (AP) sites as predominant lesions.

The current observation of UA-induced *hprt* mutations is consistent with previous reports of soluble uranyl causing cellular and genetic damages in mammalian cells. Uranyl nitrate caused cell death, micronuclei formation, chromosomal aberrations and sister chromatid exchange in CHO cells (25). It caused an increase in dicentric chromosomes (26), oxidative damage in the presence of $H_2O_2$ (27) and micronuclei formation (28) in human osteoblast cells. Uranyl chloride increased sister chromatid exchange and transformed human osteoblast cells (29) and induced apoptosis in mouse J774 macrophages (30). The current work has shown that UA will induce mutations in the DNA repair-deficient CHO EM9 line. This is the first report of mutations produced by direct exposure to UA, but it is also consistent with previous work showing mutations in the Ames Salmonella reversion assay with exposure to urine from rats with imbedded DU pellets (31) and a slight increase in *hprt* mutations in lymphocytes of Gulf war veterans with imbedded DU shrapnel (9).

UA appears to be a weak mutagen compared to other chemicals that have been tested in CHO cells by this assay, producing an average induced mutant frequency (AIMF) of $31/10^6$ surviving cells in the EM9 line (Table I). Other agents induce much higher AIMF in repair-proficient cells. Methamphetamine showed an AIMF of $13/10^6$ in the CHO K1 line (32). The dietary supplement chromium picolinate produced an AIMF of $58/10^6$ surviving cells in the AA8 line (14). $^{60}$Co γ rays produced $116/10^6$ surviving cells in the AA8 line (33). Stronger responses were observed with the alkylating agents ethyl methanesulfonate (20 mM, 1 h) and *N*-ethyl-*N*-nitrosourea (1.5 mM, 1 h), both exposures producing ~1000 mutants/$10^6$ surviving cells (34). The alkylating agent *N*-*n*-butyl-*N*-nitrosourea (2 mM, 1 h) was less mutagenic, producing ~410 mutants/$10^6$ survivors (35). However, the low mutant frequency of UA at the *hprt* locus measured in the current study may underrepresent UA mutagenicity since this assay would not detect mutants harboring multilocus mutations. Large multilocus mutations may inactivate essential genes neighboring *hprt*, causing lethality in those mutant cells since there is no homologous X chromosome to supply the essential gene. This interpretation is consistent with the observation that multiexon deletions were a dominant mutation in UA-exposed CHO EM9 cells (36).

The purpose of the current study was to begin to acquire mechanistic information. Therefore, the exposure levels in these experiments are higher than those found in drinking water. There is also evidence that cell lines may differ in their sensitivity to uranyl ion. The $CI_{50}$ of uranyl nitrate and UA in kidney cells was found to be 500–650 μM for 24 h exposures in rat, human and porcine kidney proximal tubule cell lines (37,38). However, results from a short-term MTT assay cannot be directly compared to the colony formation assay in the current study. The human osteoblast (HOS) line appears to be more sensitive to uranyl ion than the CHO line, with a 24 h exposure to 100 μM uranyl chloride producing a 0.1 survival fraction by clonogenic assay (29) versus 87% survival for this dose of UA in the current study. However, this interpretation must be tempered by the possibility that the $^{238}U/^{235}U$ isotopic ratios could vary with the different forms of depleted uranium used in these studies, with more $^{235}U$ causing more radiological toxicity. Also different relative concentrations of components in the cell culture medium for these different lines, for example carbonate or phosphate, could influence uranyl speciation, affecting uptake or bioactivity.

Uranium has generally been considered to be DNA damaging through its radioactivity, specifically through release of alpha and beta particles during its radioactive decay; however, chemical mechanisms may also exist. Combinations of depleted uranium as UA and ascorbate were found to produce DNA strand breaks in plasmid DNA that were greater than those for either UA or ascorbate alone, and were observed in the absence of ascorbate-induced reduction of uranyl ion (10), which suggested a direct chemical mechanism for uranium, ascorbate and DNA interactions because the half-lives for decay of uranium isotopes would not be changed by the addition of ascorbate.

Heavy metals in general have been considered to cause DNA damage through indirect mechanisms of free radical generation and oxidative stress. For example nickel, copper, iron and chromium are believed to either undergo electron

transfer reactions with biological reducing agents or have their redox potentials altered by chelation with biomolecules, producing a metal complex that reacts with $O_2$ or $H_2O_2$ to generate HO· or other reactive oxygen species (39–42). However, data from the current study and our previous work (10) suggest that, at least in the absence of added hydrogen peroxide, direct uranium(VI)–DNA interactions are more important than free radical mechanisms. If free radical generation were a major pathway under the current conditions, then it would have been expected that oxidative damage would have been detected by the comet assay (Figure 3).

Another mechanism by which metals damage DNA is by a direct covalent interaction. This pathway is known to be important for chromium (43), and it may occur for uranium as well. Uranium has been known to interact with DNA *in vitro* (44–46); however, to our knowledge this is the first report of U–DNA adducts recovered from cultured cells. The current experiments found that uranium covalently bonded to DNA; however, at this time data cannot distinguish between simple uranium–DNA adducts and uranium-containing DNA–protein crosslinks or uranium-containing DNA–DNA crosslinks. The observation of modest differences in adduct levels between these two cell lines is consistent with the interpretation that the CHO EM9 line is depleted in XRCC1-ligase complex, and is therefore less sensitive to crosslinks than strand breaks. Current work is in progress to measure U–DNA adducts in crosslink-sensitive lines.

The current experiments did show evidence of DNA strand breaks in CHO cells exposed to UA (Figure 3). This is consistent with other studies reporting chromosomal aberrations in CHO cells exposed to uranyl nitrate (25) and in mouse germ cells exposed to enriched uranyl fluoride (47). However, because strand breaks were not the only DNA lesion observed, it is not yet clear whether the strand breaks detected in the comet assay were caused by direct action of uranium on DNA, for example DNA hydrolysis catalyzed by uranium coordinating to the DNA phosphate backbone (10), or were indirect intermediates of DNA excision repair. Nevertheless, the lack of oxidative damage in the comet assay coupled with the presence of U–DNA adducts suggests that uranium is acting through a chemical rather than a radiological mechanism.

In conclusion, depleted uranium as UA was found to be genotoxic and mutagenic in CHO cells. The presence of U–DNA adducts lends further support to the hypothesis that uranium is chemically genotoxic. This possibility of direct U–DNA interaction should be considered when extrapolating potential risks for people exposed to uranium in the absence of measurable radioactivity, for example in soil and drinking water, and in DU-containing shrapnel.

## Acknowledgements

Funding for the study was provided by NIH grants CA096320 (DMS) and CA096281 (RCL). The ICP-OES and comet assay equipment were purchased with funding from the Arizona Board of Regents Biotechnology and Human Welfare Program (DMS). MY was supported by the NIH Minority Student Development Program grant GM56931.

## References

1. Churchill,W. (1996) *From a Native Son: Selected Essays in Indigenism, 1985–1995*. South End Press, Cambridge, MA, p. 27.
2. Durakovic,A. (2003) Undiagnosed illnesses and radioactive warfare. *Croatian Med. J.*, 44, 520–532.
3. Mulloy,K.B., James,D.S., Mohs,K. and Kornfeld,M. (2001) Lung cancer in a nonsmoking underground uranium miner. *Environ. Health Perspect.*, 1009, 305–309.
4. Gilliand,F.D., Hunt,W.C., Pardilla,M. and Key,C.R. (2000) Uranium mining and lung cancer among Navajo men in New Mexico and Arizona, 1969–1993. *J. Occup. Environ. Med.*, 42, 278–283.
5. Chen,J., Meyerhof,D.P. and Tracy,B.L. (2004) Model results of kidney burdens from uranium intakes. *Health Phys.*, 86, 3–11.
6. Pinney,S.M., Freyberg,R.W., Levine,G.E., Brannen,D.E., Mark,L.S., Nasuta,J.M., Tebbe,C.D., Buckholz,J.M. and Wones,R. (2003) Health effects in community residents near a uranium plant at Fernald, Ohio, USA. *Int. J. Occup. Med. Environ. Health*, 16, 139–153.
7. Shields,L.M., Wiese,W.H., Skipper,B.J., Charley,P. and Benally,L. (1992) Navajo birth outcomes in the Shiprock uranium mining area. *Health Phys.*, 63, 542–551.
8. Au,W.W., Lane,R.G., Legator,M.S., Whorton,E.B., Wilkinson,G.S. and Gabehart,G.J. (1995) Biomarker monitoring of a population residing near uranium mining activities. *Environ. Health Perspect*, 103, 466–470.
9. McDiarmid,M.A., Engelhardt,S., Oliver,M. *et al.* (2004) Health effects of depleted uranium on exposed Gulf War veterans: a 10-year follow-up. *J. Toxicol. Environ. Health A*, 67, 277–296.
10. Yazzie,M., Gamble,S.L., Civitello,E.R. and Stearns,D.M. (2003) Uranyl acetate causes DNA single strand breaks *in vitro* in the presence of ascorbate (vitamin C). *Chem. Res. Toxicol.*, 16, 524–530.
11. Caldecott,K.W., Tucker,J.D., Stanker,L.H. and Thompson,L.H. (1995) Characterization of the XRCCa-DNA ligase III complex *in vitro* and its absence from mutant hamster cells. *Nucleic Acid Res.*, 23, 4836–4843.
12. Thompson,L.H. and West,M.G. (2000) XRCC1 keeps DNA from getting stranded. *Mutat. Res.*, 459, 1–18.
13. O'Neill,J.P. and Hsie,A.W. (1979) The CHO/HGPRT mutation assay: experimental procedures. In Hsie,A.W., O'Neill,J.P. and McElheny,V.K. (eds), *Banbury Report 2. Mammalian Cell Mutagenesis: The Maturation of Test Systems*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 55–69.
14. Stearns,D.M., Silveira,S.M., Wolf,K.K. and Luke,A.M. (2002) Chromium(III) tris(picolinate) is mutagenic at the hypoxanthine (guanine) phosphoribosyltransferase locus in Chinese hamster ovary cells. *Mutat. Res.*, 513, 135–142.
15. Tice,R.R., Agurell,E., Anderson,D. *et al.* (2000) Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.*, 35, 206–221.
16. Singh,N.P. (2000) Microgels for estimation of DNA strand breaks, DNA-protein crosslinks and apoptosis. *Mutat. Res.*, 455, 111–127.
17. Cantoni,O., Murray,D. and Meyn,R.E. (1987) Induction and repair of DNA single-strand breaks in EM9 mutant CHO cells treated with hydrogen peroxide. *Chem. Biol. Interact.*, 63, 29–38.
18. Martin,I.V. and MacNeill,S.A. (2002) ATP-dependent DNA ligases. *Genome Biol.*, 3, 3005.1–3005.7.
19. Leppard,J.B., Dong,Z., Mackey,Z.B. and Tomkinson,A.E. (2003) Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair. *Mol. Cell Biol.*, 23, 5919–5927.
20. Pfuhler,S. and Wolf,H.U. (1996) Detection of DNA-crosslinking agents with the alkaline comet assay. *Environ. Mol. Mutagen.*, 27, 196–201.
21. Christie,N.T., Cantoni,O., Evans,R.M., Meyn,R.E. and Costa,M. (1984) Use of mammalian DNA repair-deficient mutants to assess the effects of toxic metal compounds on DNA. *Biochem. Pharmacol.*, 33, 1661–1670.
22. Churchill,M.E., Peak,J.G. and Peak,M.J. (1991) Correlation between cell survival and DNA single-strand break repair proficiency in the Chinese hamster ovary cell lines AA8 and EM9 irradiated with 365-nm ultraviolet-A radiation. *Photochem. Photobiol.*, 53, 229–236.
23. Churchill,M.E., Peak,J.G. and Peak,M.J. (1991) Repair of near-visible- and blue-light-induced DNA single-strand breaks by the CHO cell lines AA8 and EM9. *Photochem. Photobiol.*, 54, 639–644.
24. Thompson,L.H., Brookman,K.W., Dillehay,L.E., Carrano,A.V., Mazrimas,J.A., Mooney,C.L. and Minkler,J.L. (1982) A CHO-cell strain having hypersensitivity to mutagens, a defect in DNA strand-break repair, and an extraordinary baseline frequency of sister-chromatid exchange. *Mutat. Res.*, 95, 427–440.
25. Lin,R.H., Wu,L.J., Ching,H.L. and Lin-Shiau,S.Y. (1993) Cytogenic toxicity of uranyl nitrate in Chinese hamster ovary cells. *Mutat. Res.*, 319, 197–203.
26. Miller,A.C., Xu,J., Stewart,M., Brooks,K., Hodge,S., Shi,L., Page,N. and McClain,D. (2002a) Observation of radiation-specific damage in human cells exposed to depleted uranium: dicentric frequency and neoplastic transformation as endpoints. *Radiat. Prot. Dosim.*, 99, 275–278.

27. Miller,A.C., Stewart,M., Brooks,K., Shi,L. and Page,N. (2002b) Depleted uranium-catalyzed oxidative DNA damage: absence of significant alpha particle decay. *J. Inorg. Biochem.*, **91**, 246–252.
28. Miller,A.C., Brooks,K., Stewart,M., Anderson,B., Shi,L., McClain,D. and Page,N. (2003) Genomic instability in human osteoblast cells after exposure to depleted uranium: delayed lethality and micronuclei formation. *J. Environ. Radioact.*, **64**, 247–259.
29. Miller,A.C., Blakely,W.F., Livengood,D. *et al.* (1998a) Transformation of human osteoblast cells to the tumorigenic phenotype by depleted uranium-uranyl chloride. *Environ. Health Perspect.*, **106**, 465–471.
30. Kalinich,J.F., Ramakrishnan,N., Villa,V. and McClain,D.E. (2002) Depleted uranium-uranyl chloride induces apoptosis in mouse J774 macrophages. *Toxicology*, **179**, 105–114.
31. Miller,A.C., Fuciarelli,A.F., Jackson,W.E., Ejnik,E.J., Emond,C., Strocko,S., Hogan,J., Page,N. and Pellmar,T. (1998b) Urinary and serum mutagenicity studies with rats implanted with depleted uranium or tantalum pellets. *Mutagenesis*, **13**, 643–648.
32. Li,J.-H., Hu,H.-C., Chen,W.-B. and Kin,S.-K. (2003) Genetic toxicity of methamphetamine *in vitro* and in human abusers. *Environ. Mol. Mutagen.*, **42**, 233–242.
33. Diamond,A.M., Dale,P., Murray,J.L. and Grdina,D.J. (1996) The inhibition of radiation-induced mutagenesis by the combined effects of selenium and the aminothiol WR-1065. *Mutat. Res.*, **356**, 147–154.
34. Op het Veld,C.W., van Hees-Stuivenberg,S., van Zeeland,A.A. and Jansen,J.G. (1997) Effect of nucleotide excision repair on *hprt* gene mutations in rodent cells exposed to DNA ethylating agents. *Mutagenesis*, **12**, 417–424.
35. Bol,S.A., van Steeg,H., van Oostrom,C.T., Tates,A.D., Vrieling,H., de Groot,A.J., Mullenders,L.H., van Zeeland,A.A. and Jansen,J.G. (1999) Nucleotide excision repair modulates the cytotoxic and mutagenic effects of N-n-butyl-N-nitrosourea in cultured mammalian cells as well as in mouse splenocytes *in vivo*. *Mutagenesis*, **14**, 317–322.
36. Coryell,V.H. and Stearns,D.M. (2005) Molecular analysis of *hprt* mutations generated bin Chinese hamster ovary EM9 cells by uranyl acetate, by hydrogen peroxide and spontaneously. *Molec. Carcinogen.*, in press.
37. Carrière,M., Avoscan,L., Collins,R., Carrot,F., Khodja,H., Ansoborlo,E. and Gouget,B. (2004) Influence of uranium speciation on normal rat kidney (NRK-52E) proximal cell cytotoxicity. *Chem. Res. Toxicol.*, **17**, 446–452.
38. Mirto,H., Barrouillet,M.P., Henge-Napoli,M.H., Ansoborlo,E., Fournier,M. and Cambar,J. (1999) Influence of uranium(VI) speciation for the evaluation of *in vitro* uranium cytotoxicity on LLC-PK1 cells. *Hum. Exp. Toxicol.*, **18**, 180–187.
39. Kawanishi,S., Hiraku,Y., Murata,M. and Oikawa,S. (2002) The role of metals in site-specific DNA damage with reference to carcinogenesis. *Free Radic. Biol. Med.*, **32**, 822–832.
40. Schumann,K., Classen,H.G., Dieter,H.H., Konig,J., Multhaup,G., Rukgauer,M., Summer,K.H., Bernhardt,J. and Biesalski,H.K. (2002) Hohenheim consensus workshop: copper. *Eur. J. Clin. Nutr.*, **56**, 469–483.
41. Toyokuni,S. (1996) Iron-induced carcinogenesis: the role of redox regulation. *Free Radic. Biol. Med.*, **20**, 553–566.
42. Shi,X., Chiu,A., Chen,C.T., Halliwell,B., Castranova,V. and Vallyathan,V. (1999) Reduction of chromium(VI) and its relationship to carcinogenesis. *J. Toxicol. Environ. Health B Crit. Rev.*, **2**, 87–104.
43. O'Brien,T.J., Ceryak,S. and Patierno,S.R. (2003) Complexities of chromium carcinogenesis: role of cellular response, repair and recovery mechanisms. *Mutat. Res.*, **533**, 3–36.
44. Zobel,C.R. and Beer,M. (1961) Electron stains. I. Chemical studies on the interaction of DNA with uranyl salts. *J. Biophys. Biochem. Cytol.*, **10**, 335–346.
45. Drynov,I.D., Poletaev,A.I., Kharitonenkov,I.G. and Klimenko,S.M. (1974) The interaction of uranyl acetate with DNA. *Mol. Biol.*, **8**, 27–33.
46. Jeppesen,C. and Nielsen,P.E. (1989) Uranyl mediated photofootprinting reveals strong *E. coli* RNA polymerase—DNA backbone contacts in the +10 region of the DeoP1 promoter open complex. *Nucleic Acids Res.*, **17**, 4947–4956.
47. Hu,Q.Y. and Zhu,S.P. (1990) Induction of chromosomal aberrations in male mouse germ cells by uranyl fluoride containing enriched uranium. *Mut. Res.*, **244**, 209–214.

*Received on January 27, 2005; revised on August 11, 2005; accepted on August 14, 2005*

# Drinking Water with Uranium below the U.S. EPA Water Standard Causes Estrogen Receptor–Dependent Responses in Female Mice

*Stefanie Raymond-Whish,[1] Loretta P. Mayer,[1] Tamara O'Neal,[1] Alisyn Martinez,[1] Marilee A. Sellers,[1] Patricia J. Christian,[2] Samuel L. Marion,[2] Carlyle Begay,[2] Catherine R. Propper,[1] Patricia B. Hoyer,[2] and Cheryl A. Dyer[1]*

[1]Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona, USA; [2]Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona, USA

BACKGROUND: The deleterious impact of uranium on human health has been linked to its radioactive and heavy metal–chemical properties. Decades of research has defined the causal relationship between uranium mining/milling and onset of kidney and respiratory diseases 25 years later.

OBJECTIVE: We investigated the hypothesis that uranium, similar to other heavy metals such as cadmium, acts like estrogen.

METHODS: In several experiments, we exposed intact, ovariectomized, or pregnant mice to depleted uranium in drinking water [ranging from 0.5 µg/L (0.001 µM) to 28 mg/L (120 µM).

RESULTS: Mice that drank uranium-containing water exhibited estrogenic responses including selective reduction of primary follicles, increased uterine weight, greater uterine luminal epithelial cell height, accelerated vaginal opening, and persistent presence of cornified vaginal cells. Coincident treatment with the antiestrogen ICI 182,780 blocked these responses to uranium or the synthetic estrogen diethylstilbestrol. In addition, mouse dams that drank uranium-containing water delivered grossly normal pups, but they had significantly fewer primordial follicles than pups whose dams drank control tap water.

CONCLUSIONS: Because of the decades of uranium mining/milling in the Colorado plateau in the Four Corners region of the American Southwest, the uranium concentration and the route of exposure used in these studies are environmentally relevant. Our data support the conclusion that uranium is an endocrine-disrupting chemical and populations exposed to environmental uranium should be followed for increased risk of fertility problems and reproductive cancers.

KEY WORDS: depleted uranium, endocrine disruption, estrogen, estrogen receptor, female reproduction, heavy metal, Navajo reservation. *Environ Health Perspect* 115:1711–1716 (2007). doi:10.1289/ehp.9910 available via *http://dx.doi.org/* [Online 14 September 2007]

Uranium, the heaviest naturally occurring element, is valued for its radioactive properties. Development of nuclear weapons in the 1940s fueled the U.S. government's desire to become independent of foreign sources of U (Ball 1993; Moure-Eraso 1999; Panikkar and Brugge 2007). The U "boom" in the southwestern United States lasted from the early 1950s until the market collapsed in 1971, when the U.S. government ceased being the sole purchaser of U ore (Brugge and Goble 2002).

The majority of U mining/milling occurred in the Four Corners region of the United States where the Navajo Reservation is located. The Navajo Abandoned Mine Lands (AML) agency reclaims abandoned uranium mines (AUMs) under the authority and with funding from the Surface Mining Control and Reclamation Act of 1977 (Office of Surface Mining 1977). The Navajo AML agency has estimated that there are approximately 1,300 AUMs throughout the 27,000 square miles of the Navajo Nation (U.S. EPA 2004). About 50% of AUMs have been reclaimed [U.S. Environmental Protection Agency (EPA) 2004]. Unremediated AUMs enabled U to disperse into air, soil, water, and the food chain (Brugge and Goble 2002). A present-day example of unregulated U mining/milling is the Atlas Corporation Moab Uranium Mill Tailing (Moab, UT). Nearly 10,000 gallons of U-contaminated water seeps into the Colorado River daily (Oak Ridge National Laboratory 1998), and the adjacent surface water concentration of uranium is > 5 mg/L (Department of Energy 2005).

The largest American Indian reservation in the United States is the Navajo Nation, which is divided into 110 political units called Chapters. Within 33 Chapters, the U.S. EPA surveyed 226 water sources. Of these, 90 water sources were contaminated with U above the U.S. EPA safe drinking water level of 30 µg/L (0.126 µM). The U levels found in contaminated water sources ranged from 33.3 to 1,131 µg/L, with the highest concentration being 38 times the safe drinking water level (U.S. EPA 2004). The surveyed water sources were stock tanks, wells, and springs. Chapter officials identified the water sources as providing drinking water for residents without running water (U.S. EPA 2004). According to the 2000 U.S. census (2006), > 175,000 people live on the Navajo Reservation. At least half of these residents haul water from the nearest water source for household use (i.e., drinking water, cooking, and clothes laundering), making it a certainty that many Navajo Nation residents are exposed to unsafe levels of U.

The toxicity of U is due to its radioactive and chemical properties (Brugge et al. 2005; Taylor and Taylor 1997). U inhalation and/or ingestion leads to malignant and nonmalignant respiratory diseases, stomach and kidney cancer, kidney failure, and leukemia (Brugge et al. 2005; Roscoe et al. 1995). U's effect on the reproductive system was examined in early studies with rats fed high doses of 2% uranyl nitrate (UN). U exposure caused significant weight loss in dams, fewer litters, and fewer pups per litter (Maynard and Hodge 1949). When female rats were returned to chow diet without UN, they regained the lost body weight, but a reduction in the number of litters and pups per litter persisted, suggesting that the ovaries had been permanently damaged (Maynard and Hodge 1949). Female mice treated with uranyl acetate by gavage through gestation, parturition, and nursing had an increased number of dead young per litter (Paternain et al. 1989). It is likely that the high doses of U in these studies led to reproductive toxicity (Domingo 2001; Hindin et al. 2005).

Heavy metals exhibit estrogenic properties (Dyer 2007). Several heavy metals stimulate proliferation of MCF-7 human breast cancer cells (Brama et al. 2007; Choe et al. 2003; Martin et al. 2003; Martinez-Campa et al. 2006). Cadmium interacts with estrogen receptor-α (ER-α) (Brama et al. 2007; Martin et al. 2003) and binds to the ligand-binding domain of ER-α in cultured cells (Stoica et al. 2000). Cd stimulates estrogenic responses *in vivo* (Alonso-Gonzalez et al. 2007; Johnson et al. 2003). Ovariectomized rats injected with Cd had increased uterine

Address correspondence to C.A. Dyer, Northern Arizona University, Department of Biological Sciences, P.O. Box 5640, Building 21, Room 227, South Beaver St., Flagstaff, AZ 86011 USA. Telephone: (928) 523-6294. Fax: (928) 523-7741. E-mail: Cheryl.Dyer@nau.edu

We thank R. Audet, J. Getz, H. Miers, T. Layton, and Z. Robinson for their technical assistance.

This work was supported by National Institutes of Health grants R15 ES013481, IMSD GM 056931, U54 CA096320, and F31 CA110210 (SRW) and an American Physiological Society Porter Predoctoral Fellowship (S.R.W.).

The authors declare they have no competing financial interests.

Received 20 November 2006; accepted 13 September 2007.

weight, accelerated mammary gland growth/development, and accelerated vaginal opening (VO) (Johnson et al. 2003). Cd-induced estrogen-like responses were prevented by the antiestrogen ICI 182,780. Cd inhibits transcriptional activity of estradiol-activated rainbow trout ER in recombinant yeast (Guével et al. 2000). Cd treatment stimulates breast cancer cell proliferation by activating ER-α–dependent Akt (protein kinase B), Erk1/2 (extracellular signal-regulated kinase), and platelet-derived growth factor receptor-α (Brama et al. 2007). Although these studies demonstrate the estrogen activity of Cd, it should be noted that Silva et al. (2006) reported that Cd lacks estrogenic activity in the yeast estrogen screen assay, MCF-7 cell proliferation, or the E-SCREEN assay, and also failed to induce Src, Erk1, and Erk2 phosphorylation. In the present study we tested whether depleted U added to drinking water caused responses in the female mouse reproductive tract like those caused by the potent synthetic estrogen diethylstilbestrol (DES).

## Materials and Methods

*Animals.* We performed U exposure in intact female mice using 28-day-old immature B6C3F$_1$ mice (Harlan, Indianapolis, IN). For *in utero* U exposure experiments, we used 48-day-old male and female B6C3F$_1$ mice (Harlan). We used ovariectomized 28-day-old C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) for the prepubertal U and DES exposure experiments. Mice were housed with a 12:12 hr light/dark cycle and received water and food *ad libitum*. Control tap water tested for U using kinetic phosphorescence analysis, as described by Hedaya et al. (1997), was below the limit of detection (< 2 μg/L or < 8 pM). All protocols were approved by the University of Arizona or Northern Arizona University Institutional Animal Care and Use Committees. All mice were treated humanely with regard for alleviation of suffering in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Research, 1996).

*Treatments.* Animals were treated with UN hexahydrate (depleted U) (Sigma Chemical Co., St. Louis, MO) in drinking water.

Study 1: Impact of U exposure on ovarian follicle populations. *Experiment 1.1: U exposure in immature mice.* Mice were exposed to UN in their drinking water at milligram per liter doses based on a study using rats (Gilman et al. 1998). Immature 28-day-old B6C3F$_1$ mice drank water containing UN at 0.5, 2.5, 12.5, and 60.0 mg/L (1, 5, 25, and 120 μM, respectively; $n$ = 9–10 mice per group). After 30 days, we analyzed ovaries for changes in follicle populations.

*Experiment 1.2: Gestational and* in utero *U exposure in dams and female pups.* For *in utero* exposure, mice were given water containing UN at 0.5, 2.5, 12.5, or 60 μg/L (0.001, 0.005, 0.025, or 0.120 μM U, respectively) for 30 days prior to breeding. U dose was reduced a thousandfold to micrograms per liter to correspond to environmentally relevant concentrations. Mice were paired for breeding, and males were removed when females had vaginal plugs. Females continued to drink U-containing water at the above doses through gestation. On the day of birth, dams ($n$ = 5 mice per treatment group) and female pups ($n$ = 7–9 pups per treatment group) were euthanized and the ovaries collected for histology.

Study 2: Impact of U exposure on the female reproductive tract in the absence of endogenous estrogen. *Experiment 2.1: U exposure in ovariectomized mice.* For this study we used C57Bl/6J mice because of strain sensitivity to estrogen in the uterotrophic assay (Ashby et al. 2003). We also anticipated the use of genetically manipulated mice (e.g., ER-α knockout mice) on this genetic background (Lubahn et al. 1993). C57Bl/6J mice were ovariectomized at 28 days of age to remove the endogenous source of estrogen before VO. Seven days postsurgery, ovariectomized and intact mice were given tap water or water containing 0.19 μM DES or 0.06, 0.12, 1.20, or 12.00 μM U for 30 days ($n$ = 5–6 mice per treatment group).

*Experiment 2.2: Other estrogen-like effects of UN and dependence on ER activation.* Mice ovariectomized at 28 days of age were exposed to drinking water containing U or DES at the aforementioned concentrations for 10 days beginning at 50 days of age. Some mice ($n$ = 6–7 mice per group) concurrently received daily intraperitoneal (i.p.) injections of either sesame oil vehicle or 500 μg/kg ICI 182,780 (Tocris Coolson Ltd., Avonmouth, UK). Mice were examined daily at the same time for VO and cytology.

*Tissue collection and histology.* After exposure to DES or U, mice were euthanized and organs were collected for necropsy. Uteri were removed by dissecting inferior to the Fallopian tubes and superior to the vagina. Wet weights of ovary, uterus, kidney, liver, and spleen were normalized to total body weight. Uterine tissues were fixed in Bouin's solution, embedded in paraffin, and serially sectioned every 9 μm; every 10th section was mounted on slides. Tissue sections were deparaffinized in Citrasolve (Sigma Chemical Co.) and dehydrated in a series of ethanol baths. We used a Zeiss 435 VP scanning electron microscope and LEO32 V02.01 software (Carl Zeiss SMT Inc., Peabody, MA) to measure the height of uterine luminal epithelial cells. Forty measurements were randomly collected from each individual uterus.

Ovaries were trimmed of adhering tissue and fat and then fixed in Bouin's solution. They were transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 μm), mounted, and stained with hematoxylin and eosin. Nuclei of oogonia and primordial, small primary, large primary, secondary or growing, and healthy antral and atretic follicles were identified and counted in adult ovary every 20th section, and in pup ovary every 12th section (Mayer et al. 2004).

*Statistical analyses.* Oogonia and follicle numbers were determined in ovaries from individual mice and averaged. The means in control versus exposed mice were analyzed for significant differences by one-way analysis of

**Table 1.** Effects of UN exposure on specific ovarian follicle populations (follicle counts per ovary; mean ± SE) in B63CF$_1$ mice exposed to UN in drinking water for 30 days.

| Follicle type | Control (U < 0.002 mg/L) | UN (mg/L) 0.5 | 2.5 | 12.5 | 60.0 |
|---|---|---|---|---|---|
| Primordial | 65.55 ± 7.05 | 53.80 ± 8.26 | 37.88 ± 7.01 | 57.60 ± 13.29 | 61.60 ± 12.76 |
| Small primary | 26.22 ± 2.50 | 19.40 ± 3.03 | 18.56 ± 2.94 | 32.00 ± 3.51 | 21.78 ± 2.81 |
| Large primary | 12.66 ± 0.69 | 6.50 ± 1.17* | 7.44 ± 1.27* | 12.00 ± 1.51 | 9.11 ± 0.65 |
| Secondary or growing | 26.44 ± 1.08 | 24.20 ± 2.09 | 21.22 ± 1.85 | 33.30 ± 1.92* | 26.78 ± 0.81 |
| Healthy antral | 31.22 ± 2.56 | 31.00 ± 3.49 | 28.22 ± 3.71 | 29.00 ± 2.39 | 23.11 ± 2.78 |
| Atretic antral | 17.22 ± 1.37 | 15.50 ± 2.37 | 11.44 ± 1.70 | 16.00 ± 3.26 | 12.53 ± 1.37 |

$n$ = 6 per group.
*Significantly different from control ($p$ < 0.05, Tukey-Kramer post hoc test).

**Table 2.** Effects of UN exposure on body weight and tissue weight in B63CF$_1$ mice exposed to UN in drinking water for 30 days.

| Treatment | Body weight | Ovary | Uterus | Liver | Adrenal | Kidney | Spleen |
|---|---|---|---|---|---|---|---|
| Control (< 2 μg/L U) | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| U (mg/L) | | | | | | | |
| 0.5 | 101.2 | 77.5 | 97.1 | 94.2 | 95.5 | 96.0 | 104.0 |
| 2.5 | 100.4 | 72.5 | 81.8 | 94.4 | 88.4 | 91.7* | 89.9 |
| 12.5 | 104.1 | 73.9 | 115.9 | 99.2 | 120.8 | 100.9 | 103.6 |
| 60.0 | 104.6 | 62.4 | 127.8 | 110.6 | 108.5 | 94.2* | 109.8 |

Tissue weights are expressed as a percent of control values normalized to total body weight.
*Significantly different from control ($p$ < 0.05).

variance (ANOVA) with significance set at $p < 0.05$. We used Tukey-Kramer post hoc tests where appropriate. For mice exposed for 10 and 30 days, organ weights were determined for each individual within each experiment and averaged for each exposure group. In the 30-day–exposure group, uterine luminal epithelial cell height measurements were collected from individual mice and averaged for each exposure group. Additionally, in the 10-day–exposure group, VO was determined for each individual and averaged for the exposure group. The means for control versus exposed mice for organ weights, uterine epithelial cell height, and VO were analyzed for significant differences by one-way ANOVA with significance set at $p < 0.05$. We used Dunnet's post hoc test where appropriate. The means of uterine weights in controls or in mice exposed to ICI 182,780, U, or DES were analyzed by two-way ANOVA with significance set at $p < 0.05$. Persistent presence of cornified vaginal cells was determined for each individual mouse in the 10-day–exposure group. Presence and absence of cornified cells was analyzed by chi-square test with significance set at $p < 0.05$. Statistical significance of persistent presence of cornified cells was analyzed by Fisher's exact test with significance set at $p < 0.05$.

## Results

*Study 1: Impact of U exposure on ovarian follicle populations.* Experiment 1.1: U exposure in immature mice. Experiment 1.1 showed that U targets early stage ovarian follicles. As shown in Table 1, there were significantly fewer large primary follicles at 0.5 and 2.5 mg/L UN and significantly more secondary or growing follicles at 12.5 mg/L UN. However, we found no significant increase in the number of atretic follicles or decrease in healthy follicles. Because UN exposure caused a selective change in ovarian follicle populations and because there were more growing follicles at 12.5 mg/L UN, the changes could not be caused by heavy metal toxicity.

This experiment also showed that U does not lead to overt organ toxicity. We found no gross anomalies in any major organs, and body weight did not significantly change with UN exposure at any concentration. As shown in Table 2, kidney weight was significantly reduced at doses of 2.5 and 60.0 mg/L UN, but this was not surprising given the nephrotoxicity of U (Brugge et al. 2005; Taylor and Taylor 1997). These data support the conclusion that there was no systemic UN-mediated toxicity.

We found an interesting, but not statistically significant, trend of increased uterine weight at 12.5 and 60.0 mg/L UN (Table 2). We did not determine estrous cycle stage in mice at sacrifice, thus uterine weights could not be grouped relative to stage.

Experiment 1.2: Gestational and *in utero* U exposure in dams and female pups. Experiment 1.2 showed that *in utero* uranium exposure reduces pup ovary primordial follicles. As shown in Figure 1A, mice exposed to UN for 30 days before mating and through gestation had a significant reduction of small primary follicles at UN concentrations of 0.005, 0.025, and 0.120 µM compared with control mice. All other follicle populations, including primordial, secondary/growing, healthy, and atretic, were unchanged (data not shown). Neonatal mouse ovaries have only oogonia and primordial follicles. We found no difference in the number of pup ovary oogonia among control and UN exposure groups (data not shown). Primordial follicle numbers were reduced in ovaries of pups whose dams consumed water with 0.001- or 0.120-µM UN, compared with primordial follicles in pup ovaries from dams drinking control tap water (Figure 1B).

*Study 2: Impact of U exposure on the female reproductive tract in the absence of endogenous estrogen.* Experiment 2.1: U exposure in ovariectomized mice. Experiment 2.1 showed that UN exposure induces estrogen-like changes in uterine morphology and histology. Mice exposed to UN or DES had significantly increased uterine weight at 0.120 µM U and 0.19 µM DES, 3.6 and 3.8 times greater, respectively, compared with mice drinking control tap water (Figure 2A). We normalized uterine weights to body weights, which were unchanged across treatment groups. Uterine weights were not increased in ovary-intact, age-matched mice that drank U-containing water (data not shown).

Experiment 2.2: Other estrogen-like effects of UN and their mediation through ER activation. Experiment 2.2 showed that UN-mediated estrogen-like actions are blocked by concomitant exposure to an ER antagonist. To determine if the U-mediated uterotrophic response was dependent on ER activation, ovariectomized mice drinking UN-containing water were injected daily with the antiestrogen ICI 182,780. In a pilot experiment, we determined that 10 days of exposure to UN in drinking water caused a significant increase in uterine weight compared with mice drinking tap water (data not shown). Ten days of concomitant ICI 182,780 treatment blocked both UN- and DES-mediated increases in uterine weights (Figure 2B): 0.06 µM U alone, 1,070 ± 386 mg/kg total bw; 0.06 µM U plus ICI 182,780, 220 ± 28.1 mg/kg total bw; 0.19 µM DES alone, 1,530 ± 282 mg/kg total bw; 0.19 µM DES plus ICI 182,780, 252 ± 24.7 mg/kg total bw. Uterine weights of control mice were

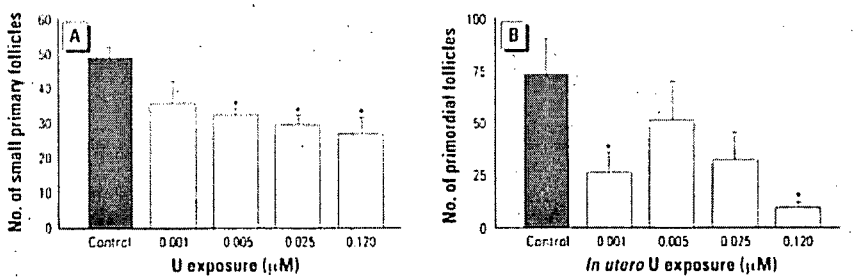



**Figure 1.** Effects of UN at 0.5, 2.5, 12.5, or 60 µg/L (0.001, 0.005, 0.025, or 0.120 µM U, respectively) on dam follicle populations and *in utero* exposed pup ovary primordial follicles. B6C3F$_1$ dams were exposed to control tap water or U in drinking water for 30 days before mating and through gestation. Ovaries from dams (*A*) and pups (*B*) were removed on the day of birth. Values shown are mean ± SE ($n$ = 7–11).
*Significantly different compared with controls ($p < 0.05$, ANOVA).

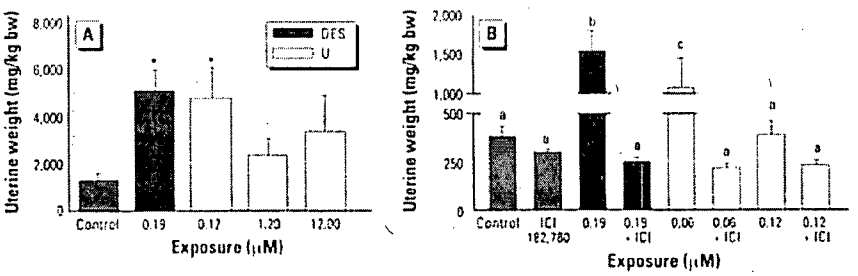



**Figure 2.** Effect of UN or DES alone and in combination with ICI 182,780 on uterine weight in ovariectomized C57Bl/6J mice. (*A*) Uteri were removed after 30 days of exposure, and wet weights were recorded and normalized to body weight; values shown are mean ± SE ($n$ = 5–6). (*B*) Uteri were removed after 10 days of exposure, and wet weights were recorded and normalized to body weight; values shown are mean ± SE ($n$ = 6–7). Different letters (a, b, c) indicate significant differences among exposure groups ($p < 0.005$).
*Significantly different compared with other exposure groups ($p < 0.001$).

not significantly different from controls treated with ICI 182,780 (Figure 2B).

One aspect of the uterotrophic response to estrogen is proliferation of the epithelial cell lining of the uterus (Kang et al. 1975; O'Brien et al. 2006). Uterine epithelial cell height was significantly greater in mice drinking water containing U or DES for 30 days (Figure 3A); 0.120 µM U, 31.01 ± 1.89 µm; 1.20 µM U: 23.79 ± 0.93 µm; 0.19 µM DES, 40.2 ± 1.85 µm; controls, 15.24 ± 0.77 µm. Figures 3B (control), 3C (0.19 µM DES), and 3D (0.12 µM U) show scanning electron micrographs illustrating changes in uterine luminal epithelial cell height. Arrows in in Figure 3C and 3D indicate pseudostratified columnar morphology typical of proliferating epithelial cells due to DES or UN exposure, respectively.

*Effects of U on VO and vaginal cell cornification.* Estrogen and endocrine-disrupting chemicals (EDCs) accelerate VO in mice (Markey et al. 2001). Ovariectomized mice exposed to 0.12 µM UN or 0.19 µM DES exhibited significantly accelerated VO (both at 52.5 days), compared with control mice (54 days) (Figure 4A). UN- or DES-mediated acceleration of puberty onset, as indicated by day of VO, was prevented by concomitant treatment with the antiestrogen ICI 182,780 (Figure 4A).

Another indication of estrogenic influence on the female reproductive tract is the persistent presence of cornified cells in vaginal smears (Gordon et al. 1986). As shown in Figure 4B, mice exposed to 0.06 µM UN (4 mice) or 0.12 µM UN (5 mice), or 0.19 µM DES (6 mice) had persistent presence of cornified vaginal cells compared with control mice (0 mice). Coincident treatment with ICI 182,780 prevented the presence of cornified vaginal cells (0.06 µM UN, 0 mice; 0.12 µM UN, 0 mice; 0.19 µM DES, 1 mouse).

## Discussion

The major contribution of the present study is the discovery that U, similar to other heavy metals, has estrogenic activity (Alonso-Gonzalez et al. 2007; Brama et al. 2007; Choe et al. 2003; Dyer 2007; Johnson et al. 2003; Martin et al. 2003; Martinez-Campa et al. 2006). To our knowledge, this has not been demonstrated before. Immature animals exposed to U in drinking water had increased uterine weight and uterine luminal epithelial cell growth, selective reduction of ovarian primary follicles but more growing follicles, accelerated VO, and persistent presence of cornified vaginal cells. U-mediated responses were blocked by coadministration of the antiestrogen ICI 181,720, indicating that an activated ER was necessary. In addition, transplacental exposure to U caused fewer primordial follicles in developing pup ovaries. These observations support the conclusion that U acts like estrogen in the female mouse reproductive tract.

U caused estrogenic responses at or below the U.S. EPA safe drinking water level of 30 µg/L (0.126 µM) (U.S. EPA 2006). The U.S. EPA safe drinking water level equals the concentration of elemental U and is 47.4% of UN dissolved in water. Therefore, the highest UN concentration of 60 mg/L equals 28 mg/L of elemental U. At first, we used milligram per liter amounts of UN in the drinking water because we expected U to cause ovarian chemical toxicity as previously reported (Maynard and Hodge 1949). Unexpectedly, at milligram per liter concentrations, U targeted only large primary follicles, causing a reduction in their number but an increase in growing follicles. At the same time, there was a trend of increasing uterine weight with increasing U dose. These results led us to determine whether U could mimic estrogen's effects on the female reproductive system. Subsequently, we analyzed uterotrophic responses in ovariectomized mice using environmentally relevant U concentrations. We observed significant effects of U on the female reproductive system at or below the U.S. EPA safe levels.

The U levels used in these experiments are well within the range of U concentrations measured in numerous water sources on the Navajo Reservation, where concentrations > 1 mg/L have been reported (Brugge and Goble 2002; U.S. EPA 2004). The Navajo Reservation is a vast expanse of primarily rural and open range land. At least half of the households on the Navajo Reservation rely on water hauled from the nearest source for household use (U.S. Census 2006). Given the frequency of water supplies with unsafe U content, there is no doubt that many of the 175,000 residents living on the Navajo Reservation are exposed to hazardous levels of U in their water (Brugge et al. 2007; Pasternak 2006).

Adult mice exposed to U while immature had fewer primary follicle populations but more secondary follicles. 17β-Estradiol ($E_2$) inhibits mouse oocyte nest breakdown and follicle assembly (Chen et al. 2007). U, mimicking $E_2$ action, may have reduced follicle assembly leading to fewer primary follicles. Dam ovaries had fewer small primary follicles at a 1,000-fold lower U concentration than did the adult nonpregnant mice, which had no significant decrease in primary follicles. The pregnant dam ovaries may have been more sensitive to U because of an up-regulation of ERs that occurs during pregnancy (Spong et al. 2000). Estrogen prevents early follicle assembly (Chen et al. 2007) but stimulates secondary or growing follicles (Drummond 2006). U exposure may have reduced primary follicle populations and stimulated growing follicles via its estrogen-like activity.

Developing embryos are exquisitely sensitive to chemical influences. U concentrations of 0.001 or 0.120 µM in the dams' drinking water led to a significant reduction in the number of primordial follicles in pup ovaries. Gestational DES exposure is linked to fewer primordial follicles in pups, resulting in fewer ovulated ova (McLachlan et al. 1982). The long-term consequence of fewer primordial follicles would lead to accelerated ovarian failure, resulting in an earlier menopause onset (Chen et al. 2007). The change in pup ovary primordial follicles with uranium dose was an inverted U-shaped curve. Inverted U-shaped curves are seen in responses resulting from *in utero* exposure to $E_2$ (Welshons et al. 2003).

The rodent uterotrophic assay is used to identify putative EDCs. Exposure to chemicals

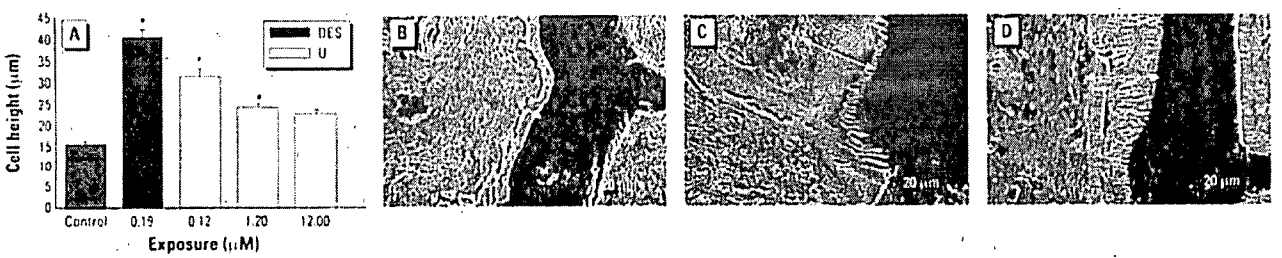



Figure 3. Uterine luminal epithelial cell growth in ovariectomized C57Bl/6J mice stimulated by UN or DES in drinking water for 30 days. (*A*) Cell height in uteri collected and prepared for scanning electron microscopy; values shown are mean ± SE ($n$ = 5 uteri at 40 measurements from each tissue). Representative scanning electron microscopy images at the same magnification of uterine epithelial cell layers from tap water control (*B*), 0.19 µM DES (*C*), or 0.12 µM U (*D*). Arrows highlight epithelial cell height in DES-exposed (*C*) and U-exposed (*D*) ovariectomized mice.

*Significantly different compared with control ($p$ < 0.0001).

with estrogenic activity are analyzed in immature rodents or ovariectomized mature rodents (Markey et al. 2001; Owens and Ashby 2002; Padilla-Banks et al. 2001). In our first experiment, the mice were immature at the outset but became sexually mature during the 30-day exposure to U. These mice exhibited a trend of increased uterine weight. If these mice had been examined for estrous stage at sacrifice, the uterine weights could have been grouped by stage, possibly enabling the trend to reach statistical significance. We used ovariectomized mice to avoid the confounding effect of estrous cycling to test whether UN caused uterotrophic responses.

The uterotrophic assay measures the consequences of three coordinated responses to estrogen or a chemical that acts like estrogen: epithelial cell growth, hyperemia, and fluid accumulation or imbibition (O'Brien et al. 2006). DES stimulation of uterine epithelial cell growth, in addition to employing classical ER-α, may also use tethered or nonclassical pathways to induce mitogenic uterine responses (O'Brien et al. 2006). This suggests that U does not need to directly activate the classical ER for uterine epithelial-cell growth.

The U dose response was not monotonic in either the uterotrophic assay or in increased uterine epithelial cell height. Many EDCs elicit low-dose responses resulting in U-shaped or inverted U-shaped dose–response curves (Myers and Hessler 2007; Welshons et al. 2003). Nonmonotonic response occurs when a xenoestrogenic compound exerts direct effects by mimicking estradiol or indirect effects by interfering with ERs or estradiol production and metabolism. Further, xenoestrogenic responses may activate or inhibit different genes at various doses, which may result in different outcomes for target end points examined at the same time points (Coser et al. 2003).

Mice exposed to U for 30 days had a more pronounced uterotrophic response than mice exposed for 10 days. This raises questions about how U may be getting into cells/tissues and by which mechanism U interacts with the ER. U enters brain endothelial cells (Dobson et al. 2006), and via specialized transport it enters polarized epithelial LLC-$PK_1$ cells (Muller et al. 2006). Vidaud et al. (2007) examined the possibility of apotransferrin transporting U into the cell. U binds to transferrin, but conformational changes do not enable transferrin receptor recognition of the U-transferrin complex, ruling out this pathway for U to enter the cell. Other ways that U may enter the cell have not been investigated: divalent metal transporter-1 (DMT-1) or calcium channels. DMT-1 functions to transport iron and other metal ions across the plasma membrane, and is ubiquitous in plants, insects, microorganisms, and vertebrates (Mims and Prchal 2005). U displaces calcium in the bone matrix (Neuman et al. 1949); therefore, it is plausible that U may use calcium channels to enter the cell. The manner and rate by which U gets into the cell may be impeded by U speciation or tissue concentration, which could result in delayed responses, as we observed with uterine weight changes after 10-day exposure compared with 30-day exposure.

Similar to DES, U accelerated VO and stimulated persistent vaginal cornified cells, which represents a constant estrus state elicited by estrogen. U-stimulated uterine and vaginal responses were blocked by ICI 182,780, indicating that ER activation was necessary but not sufficient for U to act. We have yet to define the molecular mechanisms of action by which U evokes estrogenic responses. It is possible that U may elicit estrogen-like responses as Cd is reported to, by binding the ligand binding domain of the ER (Stoica et al. 2000). As mentioned above, U estrogenic stimulation may be the result of U binding some other factor whose responses are "tethered" to the ER pathway, resulting in cross-talk that induces estrogenic responses. In summary, the stimulatory effects of U on cells of the the ovary, uterus, and vagina suggest that U acts like estrogen in the female reproductive system and is an EDC.

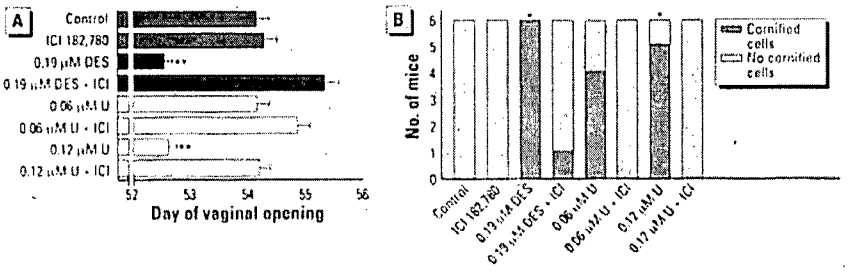



**Figure 4.** Effect of UN in drinking water on VO and presence of cornified vaginal cells. Ovariectomized C57Bl/6J mice (50 days of age) were exposed to control tap water, 0.19 μM DES, or 0.06 or 0.12 μM U for 10 days, or one of these doses plus vehicle or 500 μg/kg ICI 182,780 in vehicle. (*A*) Mice were examined daily for VO from 50 days of age to the day of vaginal opening; values shown are mean day of VO ± SE ($n$ = 6–7). (*B*) Vaginal cell cornification determined from vaginal smears collected daily; the presence and absence of vaginal cornified cells were analyzed by chi-square test ($p < 0.05$).

*Statistically significant compared with control ($p < 0.05$ by Fisher's exact test). **Significantly different from control ($p < 0.001$).

There are few reports relating environmental U exposure to reproductive health outcomes in the Four Corners region. However, in one study, a statistically significant relationship was found between birth defects and the mother's proximity to U tailings (Shields et al. 1992). In another study, the incidence of reproductive or gonadal cancer in New Mexico Native American children and teenagers is 8-fold greater than that in age-matched non-Native American individuals (Duncan et al. 1986). Environmental estrogens such as DES or bisphenol A may contribute to occurrence of reproductive anomalies and cancer later in life (Maffini et al. 2006; Newbold et al. 2006). Given our results that U is an EDC, health problems may result from inappropriate concentration or timing of exposure to this estrogen mimic.

## References


Alonso-González C, González A, Mazarrasa O, Güezmes A, Sánchez-Mateos S, Martínez-Campa C, et al. 2007. Melatonin prevents the estrogenic effects of sub-chronic administration of cadmium on mice mammary glands and uterus. J Pineal Res 42:403–410.

Ashby J, Owens W, Odum J, Tinwell H. 2003. The intact immature rodent uterotrophic bioassay: possible effects on assay sensitivity of vomeronasal signals from male rodents and strain differences. Environ Health Perspect 111:1568–1570.

Ball H. 1993. Cancer factories: America's tragic quest for uranium self-sufficiency. Contrib Med Stud 37:1–168.

Brama M, Gnessi L, Basciani S, Cerulli N, Politi L, Spera G, et al. 2007. Cadmium induces mitogenic signaling in breast cancer cell by an ERα-dependent mechanism. Mol Cell Endocrinol 264:102–108.

Brugge D, de Lemos JL, Bui C. 2007. The Sequoyah Corporation fuels release and the Church Rock spill: unpublicized nuclear releases in American Indian communities. Am J Public Health 97:1595–1600.

Brugge D, deLemos JL, Oldmixon B. 2005. Exposure pathways and health effects associated with chemical and radiological toxicity of natural uranium: a review. Rev Environ Health 20:177–193.

Brugge D, Goble R. 2002. The history of uranium mining and the Navajo people. Am J Public Health 92:1410–1419.

Chen Y, Jefferson WN, Newbold RR, Padilla-Banks E, Pepling ME. 2007. Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary *in vitro* and *in vivo*. Endocrinology 148:3580–3590.

Choe SY, Kim SJ, Kim HG, Lee JH, Choi Y, Lee Y, et al. 2003. Evaluation of estrogenicity of major heavy metals. Sci Total Environ 312:15–21.

Coser KR, Chesnes J, Hur J, Ray S, Isselbacher KJ, Shioda T. 2003. Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/BUS breast cancer cells by DNA microarray. Proc Natl Acad Sci USA 100:13994–13999.

Department of Energy. 2005. Remediation of the Moab Uranium Mill Tailings, Grand and San Juan Counties, Utah, Final Environmental Impact Statement. DOE/EIS-0355. Available: http://www.ch.doe.gov/nepa/eis/eis0355 [accessed 29 August 2007].

Dobson AW, Lack AK, Erikson KM, Aschner M. 2006. Depleted uranium is not toxic to rat brain endothelial (RBE4) cells. Biol Trace Elem Res 110:61–72.

Domingo JL. 2001. Reproductive and developmental toxicity of natural and depleted uranium: a review. Reprod Toxicol 15:603–609.

Drummond AE. 2006. The role of steroids in follicular growth. Reprod Biol Endocrinol 4:16; doi:10.1186/1477-7827-4-16 [Online 10 April 2006].

Duncan MH, Wiggins CL, Samet JM, Key CR. 1986. Childhood cancer epidemiology in New Mexico's American Indians.

Hispanic whites, and non-Hispanic whites, 1970–82. J Natl Cancer Inst 76:1013–1018.

Dyer CA. 2007. Heavy metals as endocrine disrupting chemicals. In: Endocrine-Disrupting Chemicals: From Basic Research to Clinical Practice (Gore AC, ed). Totowa, NJ:Humana Press, 111–133.

Gilman AP, Villeneuve DC, Secours VE, Yagminas AP, Tracy BL, Quinn JM, et al. 1998. Uranyl nitrate: 28-day and 91-day toxicity studies in the Sprague-Dawley rat. Toxicol Sci 41:117–128.

Gordon MN, Osterburg HH, May PC, Finch CE. 1986. Effective oral administration of 17β-estradiol to female C57BL/6J mice through drinking water. Biol Reprod 35:1088–1095.

Gueguet RL, Petit FG, Goff PL, Métivier R, Valotaire Y, Pakdel F. 2000. Inhibition of rainbow trout (*Oncorhynchus mykiss*) estrogen receptor activity by cadmium. Biol Reprod 62:255–266.

Hedaya MA, Birkenfeld HP, Kathren RL. 1997. A sensitive method for the determination of uranium in biological samples utilizing kinetic phosphorescence analysis (KPA). J Pharm Biomed Anal 15:1157–1165.

Hindin R, Brugge D, Panikkar B. 2005. Teratogenicity of depleted uranium aerosols: a review from an epidemiological perspective. Environ Health 4:17; doi:10.1186/1476-069X-4-17 [Online 26 August 2005].

Institute of Laboratory Animal Research. 1996. Guide for the Care and Use of Laboratory Animals. Washington, DC:National Academy Press.

Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G, et al. 2003. Cadmium mimics the *in vivo* effects of estrogen in the uterus and mammary gland. Nat Med 9:1081–1084.

Kang YH, Anderson WA, DeSombre ER. 1975. Modulation of uterine morphology and growth by estradiol-17β and an estrogen antagonist. J Cell Biol 64:682–691.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166.

Maffini MV, Rubin BS, Sonnenschein C, Soto AM. 2006. Endocrine disruptors and reproductive health: the case of bisphenol-A. Mol Cell Endocrinol 254–255:179–186.

Markey CM, Michaelson CL, Veson EC, Sonnenschein C, Soto AM. 2001. The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A. Environ Health Perspect 109:55–60.

Martin MB, Reiter R, Pham T, Avellanet YR, Camara J, Lahm M, et al. 2003. Estrogen-like activity of metals in MCF-7 breast cancer cells. Endocrinology 144:2425–2436.

Martinez-Campa C, Alonso-González C, Mediavilla MD, Cos S, González A, Ramos S. 2006. Melatonin inhibits both ERα activation and breast cancer cell proliferation induced by a metallo-estrogen, cadmium. J Pineal Res 40:291–296.

Mayer LP, Devine PJ, Dyer CA, Hoyer PB. 2004. The follicle-deplete mouse ovary produces androgen. Biol Reprod 71:130–138.

Maynard EA, Hodge HC. 1949. Studies of the toxicity of various uranium compounds when fed to experimental animals. In: Pharmacology and Toxicology of Uranium Compounds (Voegtlin C, Hodge HC, eds). New York:McGraw-Hill Book Company, Inc., 309–376.

McLachlan JA, Newbold RR, Shah HC, Hogan MD, Dixon RL. 1982. Reduced fertility in female mice exposed transplacentally to diethylstilbestrol (DES). Fertil Steril 38:364–371.

Mims MP, Prchal JT. 2005. Divalent metal transporter 1. Hematology 10:339–345.

Moure-Eraso R. 1999. Observational studies as human experimentation: the uranium mining experience in the Navajo Nation (1947–66). N Solutions: J Environ Occup Health Policy 9:163–178.

Muller D, Houpert P, Cambar J, Hengé-Napoli M-H. 2006. Role of the sodium-dependent phosphate co-transporters and of the phosphate complexes of uranyl in the cytotoxicity of uranium in LLC-$PK_1$ cells. Toxicol Appl Pharmacol 214:166–177.

Myers P, Hessler W. 2007. Does "the Dose Make the Poison"? Extensive Results Challenge a Core Assumption in Toxicology. Available: http://www.environmentalhealthnews.org/sciencebackground/2007/2007-0415nmdrc.html [accessed 29 August 2007].

Neuman WF, Neuman MW, Main ER, Mulryan BJ. 1949. The deposition of uranium in bone: VI. Ion competition studies. J Biol Chem 179:341–348.

Newbold RR, Padilla-Banks E, Jefferson WN. 2006. Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. Endocrinology 147:S11–S17.

Oak Ridge National Laboratory. 1998. Tailings Pile Seepage Model The Atlas Corporation Moab Mill, Moab, Utah. Grand Junction, CO:Oak Ridge National Laboratory, Environmental Technology Section. Available: http://www.ornl.gov/~webworks/cpprt/y2001/rpt/112413.pdf [accessed 29 August 2007].

O'Brien JE, Peterson TJ, Tong MH, Lee EJ, Pfaff LE, Hewitt SC, et al. 2006. Estrogen-induced proliferation of uterine epithelial cells is independent of estrogen receptor α binding to classical estrogen response elements. J Biol Chem 281:26683–26692.

Office of Surface Mining. 1977. Surface Mining Control and Reclamation Act of 1977. Public Law 95-87. Available: http://www.osmre.gov/smcra.htm [accessed 29 August 2007].

Owens JW, Ashby J. 2002. Critical review and evaluation of the uterotrophic bioassay for the identification of possible estrogen agonists and antagonists: in support of the validation of the OECD uterotrophic protocols for the laboratory rodent. Crit Rev Toxicol 32:445–520.

Padilla-Banks E, Jefferson WN, Newbold RR. 2001. The immature mouse is a suitable model for detection of estrogenicity in the uterotrophic assay. Environ Health Perspect 109:821–826.

Panikkar B, Brugge D. 2007. The ethical issues in uranium mining research in the Navajo Nation. Account Res 14:121–153.

Pasternak J. 2006. Blighted homeland. A peril dwelt among the Navajos. Los Angeles Times (Los Angeles, CA), 19 November. Available: http://www.latimes.com/news/printedition/front/la-na-navajo19nov19,1,4865021.full.story [accessed 4 September 2007].

Paternain JL, Domingo JL, Ortega A, Llobet JM. 1989. The effects of uranium on reproduction, gestation, and postnatal survival in mice. Ecotoxicol Environ Saf 17:291–296.

Roscoe RJ, Deddens JA, Salvan A, Schnorr TM. 1995. Mortality among Navajo uranium miners. Am J Public Health 85:535–540.

Shields LM, Wiese WH, Skipper BJ, Charley B, Benally L. 1992. Navajo birth outcomes in the Shiprock uranium mining area. Health Phys 63:542–551.

Silva E, Lopez-Espinosa MJ, Molina-Molina JM, Fernandez M, Olea N, Kortenkamp A. 2006. Lack of activity of cadmium in *in vitro* estrogenicity assays. Toxicol Appl Pharmacol 216:20–28.

Speng CY, McCune SK, Sternberg EM, Gustafsson JA. 2000. Maternal estrogen receptor-β expression during mouse gestation. Am J Reprod Immunol 44:249–252.

Stoica A, Katzenellenbogen BS, Martin MB. 2000. Activation of estrogen receptor-β by the heavy metal cadmium. Mol Endocrinol 14:545–553.

Taylor DM, Taylor SK. 1997. Environmental uranium and human health. Rev Environ Health 12:147–157.

U.S. Census Bureau. 2006. We the People: American Indians and Alaska Natives in the United States. Available: http://www.census.gov/prod/2006pubs/censr-28.pdf [accessed 4 September 2007].

U.S. EPA (U.S. Environmental Protection Agency). 2004. Abandoned Uranium Mines on the Navajo Nation, Arizona. EPA Region 9. Available: http://yosemite.epa.gov/r9/sfund/overview.nsf/0/c502c488f1841dc488256aee007c11bc?OpenDocument [accessed 4 September 2007].

U.S. EPA (U.S. Environmental Protection Agency). 2006. Basic Information about Radionuclides in Drinking Water. Available: http://epa.gov/safewater/radionuclides/basicinformation.html [accessed 4 September 2007].

Vidaud C, Gourion-Arsiquaud S, Rollin-Genetet F, Tome-Celer C, Plantevin S, Pible O et al. 2007. Structural consequences of binding of $UO_2^{2+}$ to apotransferrin: can this protein account for entry of uranium into human cells? Biochemistry 46:2215–2226.

Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS. 2003. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ Health Perspect 111:994–1006.

**Exhibit B**- Dr. Hannan Lagarry's Expert Report

# EXPERT OPINION REGARDING ISL MINING IN DAWES COUNTY, NEBRASKA

Hannan E. LaGarry, Ph. D.
210 North Main Street, Chadron NE 69337
hlagarry@csc.edu

## INTRODUCTION

In 2007, Chadron Creek, a stream that supplies water to the city of Chadron, Nebraska, went dry for the first time in the citys' history. Subsequent study of the creek's water flow rates by Chadron State College students suggested that normal amounts of water are flowing from the springs, but the water is disappearing into deeper alluvium or into fractures in the rock (Balmat & others 2008, Butterfield & others 2008). Following these observations, a Chadron State College graduate student began studying the widespread faults and lineaments of northwestern Nebraska using data collected by high-flying aircraft, satellites, and the space shuttle (Balmat & Leite 2008). These data, along with contributions from scientists from the Nebraska Geological Survey, the United States Geological Survey, the University of Nebraska School of Natural Resources, and the Upper Niobrara-White Natural Resource District, were presented in May 2008, at "Our Water, Our Future: a Town Hall Meeting." The consensus opinion of the presenters was that water shortages and declining water quality are real and worsening problems in northwestern Nebraska.

In January 2008 I was contacted by the Western Nebraska Resource Council for information about the geology and hydrology of northwestern Nebraska, and how uranium contamination might spread from an ISL mine site into surrounding areas. I was shown documentation reporting spills of mine waste water, and asked if there was any way that this waste could have made it's way northward 30 miles (see below). The uranium mine is situated along the same aquifers and fault zones as Chadron Creek. If faults and joints are draining the flow of Chadron Creek, they could also be allowing mine waste waters to migrate through faulted and jointed confining layers. A review of the scientific literature showed that faults and joints are well-known in some areas, but especially along the Pine Ridge near Chadron and Crawford (Swinehart & others 1985), and along the southern border of the Pine Ridge Reservation near the towns of Whiteclay, Nebraska, and Pine Ridge, South Dakota (Fielding & others 2007). Faults are also common in the vicinity of Taodstool Park on northeastern Sioux County and northwestern Dawes County (LaGarry & LaGarry 1997).

I am offering this expert opinion regarding ISL uranium mining near Crawford because I am concerned that unmapped and unmonitored faults may be transmitting lixiviant and waste water through confining layers and into the White River, the alluvium within the White River Valley, and into the secondary porosity of the Brule Formation. I am not against uranium mining in fact or principle. Since arriving in Chadron three years ago, my spouse (also a geologist) and I have sought employment at Crow Butte Resources. Crow Butte employs many of Chadron State College's recent graduates, This issue isn't even about the uranium. It's about protecting the region's water supply, and the future inhabitability of northwestern Nebraska and southwestern South Dakota. In this document, I will briefly explain the basis for my concerns, and propose a series of studies that should clarify whether or not Crow Butte Resources is contaminating the region's water, and if so, how much.

## PROFESSIONAL BACKGROUND

I have 20 years experience studying the rocks and fossils of northwestern Nebraska. From 1988-1991 I collected fossils from northern Sioux County for my dissertation work. From 1991-1996 I led field parties from the University of Nebraska State Museum while mapping the fossils and geology of the Oglala National Grassland in Sioux and Dawes Counties. From 1996-2006 I led a team of geologists from the Nebraska Geological Survey that mapped in detail the

surficial geology of most of northwestern Nebraska (a total of 80 1:24,000 quadrangles). This mapping included the entire Pine Ridge area and the area between Crawford, Nebraska and Pine Ridge, South Dakota. These maps, including digital versions (ArcInfo) and supporting field notes, are available from the University of Nebraska-Lincoln School of Natural Resources (contact James B. Swinehart). As a direct consequence of this mapping, I have published peer-reviewed articles on the Chadron Formation (Terry & LaGarry 1998), the Brule Formation (LaGarry 1998), the mapping of surficial deposits (Wysocki & others 2000, 2005), and local faults (Fielding & others 2007). Based on this mapping, we also intend to revise and reclassify the remaining rocks and surficial sediments of northwestern Nebraska.

STRATIGRAPHY OF WATER-BEARING ROCKS IN NORTHWESTERN NEBRASKA

The rocks of northwestern Nebraska range from Cretaceous to Pleistocene in age, and consist entirely of sedimentary rocks. These rocks vary in thickness and geographic extent, and are described as follows (see LaGarry & LaGarry 1997, Terry 1998).

Pierre Shale (aquiclude 1) - underlies all other units, generally 1000'-2000' thick. Contributes small amounts of sulfer and arsenic to overlying surface aquifers (e.g. modern White River alluvium) and water in streams and impoundments. Joints and faults within this unit contain minerals deposited by water movement in the geological past,

Chamberlain Pass Formation (aquifer 1) - formerly 'basal Chadron sandstone,' base of White River Group, overlies Pierre Shale, underlies Chadron Formation and modern river alluvium. Channel sandstones within this unit are a local aquifer and are mined for uranium. Water from this unit is typically used for residential and livestock supplies. Unit was deposited in an ancient paleovalley oriented generally from Crawford in the N-NW and Bayard to the S-SE. Joints and faults within this unit contain minerals deposited by water movement in the geological past,

Chadron Formation (aquitard 1) - middle of White River Group, overlies Chamberlain Pass Formation, underlies Brule Formation and modern river alluvium. Generally impermeable, except where fractured. Many faults and joints contain minerals deposited by water movement in the geologic past.

Brule Formation (aquitard 2) - top of White River Group, overlies Chadron Formation, underlies Arikaree and Ogallala groups (High Plains Aquifer) and modern river alluvium. Generally impermeable, except where fractured. Where fractured, has enough water to be included with overlying High Plains Aquifer. Used locally for residential and low-intensity agricultural supplies. Secondary porosity in Brule can transmit water up to 1500' day. Many faults and joints contain minerals deposited by water movement in the geologic past.

Arikaree Group (aquifer 3, lower part) - base of High Plains Aquifer, overlies Brule Formation of the White River Group, underlies Ogallala Group and modern river alluvium. Consists of moderately porous and permeable sandstones and silty sandstones. Coarser sandstone beds deposited along preexisting fault traces. Unit highly faulted and jointed along Pine Ridge Escarpment. Water supplies springs that feed local creeks, and is used for high-capacity irrigation wells.

Ogallala Group (aquifer 3, upper part) - upper part of High Plains Aquifer, overlies Arikaree Group, underlies modern rever alluvium and sand dunes. Consists of highly porous and permeable sandstones and conglomerates, Coarser sandstone beds deposited along preexisting fault traces. Unit highly faulted and jointed along Pine Ridge Escarpment. Water is used for high-capacity irrigation wells.

Modern river alluvium (aquifer 4) - overlies all bedrock units at one place or another. Consists of layers of silt and sand and lens-shaped ribbons of coarse gravel. Unit also overlies major fault zones. Unit is used as aquifer, and supplies water to residences, livestock, and in the case of the

White River, supplies water to the cities of Crawford, Nebraska and Pine Ridge, South Dakota, among others. Crow Butte Resources surface operations all occur on this unit.

The recent mapping of the geology of northwestern Nebraska has shown that the simplified, "layer cake" concept applied by pre-1990's workers is incorrect, and overestimates the thickness and areal extent of many units by 40-60%. Many units' distributions are heavily influenced by the contours of the ancient landscapes onto which they were deposited. For example, when considered to be the 'basal Chadron sandstone,' the Chamberlain Pass Formation was assumed to have a distribution equal to that of the overling Chadron Formation. However, the Chamberlain Pass Formation is 1-1.5 million years (Ma) older than the Chadron Formation, and has a distribution determined by the ancient topography weathered into the Pierre Shale prior to deposition of the Chamberlain Pass Formation.

SECONDARY POROSITY IN NORTHWESTERN NEBRASKA

Secondary porosity, in the form of intersecting faults and joints, is common in northwestern Nebraska, especially along the Pine Ridge Escarpment (see Swinehart & others 1985). These faults and joints are generally oriented NW-SE and SW-NE, and are most likely a result of the uplift of the Black Hills of southwestern South Dakota. The Black Hills have been tectonically active since the late Eocene (Evans & Terry 1994), and continued to fault, fracture, and fold the rocks of northwestern Nebraska and southwestern South Dakota into the middle Miocene (Fielding & others 2007). These faults and fractures transect all major bedrock units listed above. These faults could potentially connect the uranium-bearing Chamberlain Pass Formation to modern river alluvium, and connect the uranium-bearing Chamberlain Pass Formation to the overlying secondary porosity of the Brule Formation.

In addition to allowing contaminated water to move vertically, it can also transmit water horizontally. Many of the faults in northwestern Nebraska persist for tens of miles (Diffendal 1994, Fielding & others 2007). Also, many of the ancient river deposits of the Arikaree and Ogallala Groups, along with the alluvium deposited by modern rivers, follow the faults zones because fractured rock erodes more easily. Swinehart & others (1985) and Diffendal (1994) reported faults that could transmit contaminants from Crawford to Chadron, and from Crawford to Pine Ridge, South Dakota. In its license amendment for the North trend expansion, Crow Butte Resources reports a fault along the White River that could transport contaminants from the ISL mine to the White River, and from the river directly to Pine Ridge, South Dakota.

CONTAMINANT PATHWAYS

There are two principal pathways through which contaminated water could migrate away from Crow Butte Resources well fields and into adjacent areas: 1) along the White River alluvium (modern river alluvium); and 2) along faults. The White River alluvium can receive contaminants from three sources: a) from surface spills at the Crow Butte mine site; b) from waters transmitted through the Chamberlain Pass Formation where it is exposed at the land surface; and c) through faults. Contaminants within the White River can be transmitted into the areas where the alluvium intersects faults downstream from Crawford. Once into the White River alluvium, every rain event will push the contaminants a little bit downstream. In the case of the White River, downstream is to the N-NE and directly onto the Pine Ridge Reservation. Residential users, agricultural users, wildlife, and the City of Crawford all receive water supplies from the White River alluvium.

The second pathway is through faults. These faults can receive contaminants from three sources: a) from surface spills into the White River alluvium; b) from waters transmitted through the Chamberlain Pass Formation; and c) from underground excursions, which can of either lixiviant or uranium-laden water. Once into the faults, contaminants could migrate along the groundwater gradiant (which is generally eastwards) northeastward towards the Pine Ridge Reservation or southeastward toward Chadron and the majority of the remaining High Plains Aquifer. Uranium could also be drawn upwards into parts of the High Plains Aquifer by high-

capacity irrigation wells, some of which are known to be within major fault zones (northernmost Sheridan County, Nebraska).

In May of 2008, I was asked to evaluate the importance of a "whistleblower letter" from Mr. John Peterson, a mining geologist, to Mr. Gary Konwinski of the Nuclear Regulatory Commission. This letter is dated 4 April 1989, and expresses Mr. Petersons concern that information pertaining to faults was being suppressed so that that Crow Butte Resources (CBR) could mine in an unsafe area. Mr. Peterson's main contention is that the uranium mined by CBR occurs within the faults themselves, and is not a roll-front deposit as CBR maintains. This would be the worst possible situation. If there are minerals within faults, they are there because flowing water brought them there and deposited them there. If there are minerals along the faults and CBR is mining them, then they (CBR) are progressively "uncorking" the flow pathways along these faults. If this is the true situation, the risk of spilling contaminants into these faults increases with additional mining, and contamination by chemically altered waters is a virtual certainty. Also, mining the Chamberlain Pass Formation could cause these faults to move again. This could create new, unforeseen pathways for contaminants spread through.

THE PROBLEM OF ARTESIAN WATER

Artesian flow occurs along the Pine Ridge of Nebraska when there is a hydrologic connection, through faults or highly permeable strata, between the Chamberlain PassFormation and the High Plains Aquifer. The weight of water in the topographically higher High Plains Aquifer exerts pressure downward into the Chamberlain Pass Formation, which can be released as artesian water flow where the topographically lower Chamberlain Pass Formation is exposed at the surface, or where it is punctured by drilling. Artesian flow was predicted by NDEQ in their evaluation of CBR's petition for an aquifer exemption, and was observed by a local landowner as CBR did test drilling for the North Trend Expansion. Artesian flow could transmit the most mineral-laden of waters onto the land surface (and into White River alluvium) and discharge large amounts of contaminants into aquifers or faults in a very short time.

CONCLUDING REMARKS

Based on the arguments presented above, it is my expert opinion that ISL mining in the Crawford, Nebraska area should not be allowed to continue until the potential contaminant pathways of the White River alluvium and the SW-NE and NW-SE trending fault zones are examined and monitored. To this end, I suggest:

1. establishing a GIS database for the mapping of existing geologic units and features (e.g., faults). This would allow computer modeling of the region geology, hydrology, and structure, and would present the most complete picture of the data for final evaluation. Data acquired during the following investigations would be incorporated to the database.

2. map the White River alluvium in order to characterize its potential as a conduit for radioactive contaminants.

3. sample water from the White River at regular intervals (e.g., 2 miles) between Crawford and Pine Ridge to locate a plume of contaminated water or sediments, if present.

4. if contaminants are detected, convert sample wells to monitoring wells.

5. map the network of faults present in northwestern Nebraska and southwestern South Dakota.

6. pump test the faults to determine their permeability and the rate of water flow along them.

7. if water flow is detected along the faults, the convert selected sampling wells into monitoring wells.

8. color the water used in all underground stages of production. This will allow future leaks to be detected even if they manifest far from the mined area.

If these steps were taken, and the threat of uranium contamination were to be disproven, then Crow Butte Resources can proceed to mine uranium, but with renewed confidence, public trust, and regulatory credibility. If these steps were taken, and the threat of uranium contamination were to be confirmed, then it may be possible to mitigate the situation such that water supplies are protected and preserved as much as possible following the early detection of contaminants.

REFERENCES

Balmat, J. L. & M. B. Leite 2008. Identification of geological structures from lineaments on remotely sensed images of the Black Hills-Pine Ridge region. Proceedings of the 118th Annual Meeting of the Nebraska Academy of Sciences, p.28.

Balmat, J. W., J. L. Balmat, M. J. Culver, A. C. Butterfield, C. A. Kaiser, J. Zweibel, H. E. LaGarry, & M. B. Leite. 2008. Preliminary geology of the Chadron Creek watershed, Dawes County, Nebraska. Proceedings of the 118th Annual Meeting of the Nebraska Academy of Sciences, p.64.

Butterfield, A. C., J. L. Balmat, J. W. Balmat, K. Young, M. Culver, C. A. Kaiser, J. Zweibel, M. B. Leite, & H. E. LaGarry. 2008. Discharge of Chadron Creek, Dawes County, Nebraska. Proceedings of the 118th Annual Meeting of the Nebraska Academy of Sciences, pp. 64-65.

Diffendal, R. F. 1994. Geomorphic and structural features of the Alliance 1 degree X 2 degree Quadrangle, western Nebraska, discernible from synthetic-aperature radar imagery and digital shaded-relief maps. Rocky Mountain Geology; October 1994; v. 30; no. 2; p. 137-147.

Evans, J. E. & D. O. Terry, Jr. 1994. The significance of incision and fluvial sedimentation in the basal White River Group (Eocene-Oligocene), badlands of South Dakota, U. S. A. Sedimentary Geology 90:137-152.

Fielding, C. R., H. E. LaGarry, L. A. LaGarry, B. E. Bailey, & J. B. Swinehart. 2007. Sedimentology of the Whiteclay gravel beds in northwestern Nebraska, USA: structurally controlled drainage promoted by early Miocene uplift of the Black Hills Dome. Sedimentary Geology 202:58-71.

LaGarry, H. E. 1998. Lithostratigraphic revision and redescription of the Brule Formation(White River Group), northwestern Nebraska, pp. 63-92 in (D. O. Terry, Jr., H. E. LaGarry, & R. M. Hunt, Jr., eds.) Depositional environments, lithostratigraphy, and biostratigraphy of the White River and Arikaree Groups (late Eocene to early Miocene, North America). Geological Society of America Special Paper 325, 216 p.

LaGarry, H. E. & L. A. LaGarry. 1997. Geology of the Montrose, Orella, Wolf Butte, Roundtop, and Horn 7.5' USGS quadrangles, Sioux and Dawes counties, Nebraska. University of Nebraska-Lincoln Conservation and Survey Division Open-file Report 48, 161 p.

Swinehart, J. B., V. L. Souders, H. M. DeGraw, & R. F. Diffendal Jr. 1985. Cenozoic paleogeography of western Nebraska, pp. 209– 229 in (R. M. Flores & S. S. Kaplan, eds.), Cenozoic Paleogeography of West-Central United States. Proceedings of Rocky Mountain Paleogeography Symposium 3, Society of Economic Paleontologists and Mineralogists. Denver, Colorado.

Terry, D. O., Jr. & H. E. LaGarry. 1998. The Big Cottonwood Creek Member: a new member of the Chadron Formation in northwestern Nebraska, pp. 117-142 in (D. O. Terry, Jr., H. E. LaGarry, & R. M. Hunt, Jr., eds.) Depositional environments, lithostratigraphy, and biostratigraphy of the White River and Arikaree Groups (late Eocene to early Miocene, North America). Geological Society of America Special Paper 325, 216 p.

Terry, D. O., Jr. 1998. Lithostartigraphic revision and correlation of the lower part of the White River Group: South Dakota to Nebraska, pp. 15-38 in (D. O. Terry, Jr., H. E. LaGarry, & R. M. Hunt, Jr., eds.) Depositional environments, lithostratigraphy, and biostratigraphy of the White River and Arikaree Groups (late Eocene to early Miocene, North America). Geological Society of America Special Paper 325, 216 p.

Wysocki, D. A., P. J. Schoeneberger, & H. E. LaGarry. 2005. Soil Surveys: a window to the subsurface. Geoderma 126:167-180.

Wysocki, D. A., P. J. Schoeneberger, & H. E. LaGarry. 2000. Geomorphology of soil landscapes, pp. E5-E39 in (M. E. Sumner, editor-in-chief) CRC Handbook of Soil Science. Chemical Rubber Company Press.

## Hearing Docket

**From:** Elizabeth Lorina [elorina@gnzlawfirm.com]
**Sent:** Monday, July 28, 2008 11:46 PM
**To:** Hearing Docket
**Subject:** Crow Butte Resources, Docket Number 40-8943
**Attachments:** OST_Petition_completeJuly28.pdf

Please docket the attached petition to intervene.

I have installed a digital ID, but it will not recognize it when I attempted to use the e-filing system (it will not let me sign the document). The petition to intervene is attached, and I will follow up in the morning. Thank you.

Elizabeth Lorina
Gonzalez Law Firm
522 7th Street, Suite 202
Rapid City, SD 57701
605-716-6355 (telephone)
605-716-6357 (facsimile)

This e-mail is sent by a law firm and contains information that may be privileged and confidential. If you are not the intended recipient, please delete the e-mail and notify us immediately.

Received: from mail2.nrc.gov (148.184.176.43) by OWMS01.nrc.gov
(148.184.100.43) with Microsoft SMTP Server id 8.0.751.0; Mon, 28 Jul 2008
23:46:43 -0400
X-Ironport-ID: mail2
X-SBRS: 6.3
X-MID: 19542994
X-IronPort-AV: E=Sophos;i="4.31,269,1215403200";
d="pdf?scan'208,217";a="19542994"
Received: from web1003.biz.mail.sp1.yahoo.com ([216.252.120.73]) by
mail2.nrc.gov with SMTP; 28 Jul 2008 23:45:59 -0400
Received: (qmail 9694 invoked by uid 60001); 29 Jul 2008 03:45:52 -0000
Received: from [208.107.146.73] by web1003.biz.mail.sp1.yahoo.com via HTTP;
Mon, 28 Jul 2008 20:45:52 PDT
X-Mailer: YahooMailRC/975.45 YahooMailWebService/0.7.199
Date: Mon, 28 Jul 2008 20:45:50 -0700
From: Elizabeth Lorina <elorina@gnzlawfirm.com>
Subject: Crow Butte Resources, Docket Number 40-8943
To: Hearing Docket <Hearing.Docket@nrc.gov>
MIME-Version: 1.0
Content-Type: multipart/mixed; boundary="0-1415839545-1217303152=:6758"
Message-ID: <331516.6758.qm@web1003.biz.mail.sp1.yahoo.com>
Return-Path: elorina@gnzlawfirm.com