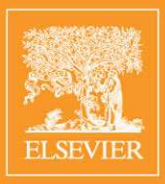

ARSENIC EXPOSURE AND HEALTH EFFECTS IV

Willard R. Chappell

Arsenic Exposure and Health Effects IV

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Arsenic Exposure and Health Effects IV

Proceedings of the Fourth International Conference on Arsenic Exposure and Health Effects, June 18–22, 2000, San Diego, California

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Preface

The Society of Environmental Geochemistry and Health (SEGH) Fourth International Conference on Arsenic Exposure and Health Effects was held June 18–22, 2000 in San Diego, California. Both public and private groups sponsored the conference. In addition to SEGH and the University of Colorado at Denver, other sponsors included the US Environmental Protection Agency (US EPA), the Electric Power Research Institute (EPRI), the International Council on Metals in the Environment (ICME), Kennecott Corporation and UNICEF.

Over 250 people, including the speakers and poster presenters, attended the 4th conference. Of these, approximately one-third were non-US citizens; the largest groups were from Asia and Europe. The attendees included scientists from academia, industry, local government, state government, the US federal government and several government scientists from outside the US. The disciplines represented and the content of the presentations included geochemistry, chemistry, molecular biology, biochemistry, epidemiology and medicine. Several outstanding papers and posters presented at the conference generated lively discussion and debate, not only about scientific issues, but also social, public policy and regulatory issues. The interactions between the attendees both during and outside the sessions resulted in many new contacts between scientists.

There were 11 platform sessions and three poster sessions with 55 speakers and 120 posters. Panel discussions were held after each session to encourage discussion and debate. One session was devoted entirely to the proposed maximum contaminant level (MCL) of 5 μg/l where EPA scientists presented the reasoning used to arrive at that recommendation. As most people know, the final MCL was proposed to be 10 μg/l, but the present administration has decided to review that recommendation before letting it be implemented. Thus, at the time of writing of this preface, the situation is unclear. The final session was a panel discussion on research needs to refine the MCL. As in past conferences, new countries reported elevated arsenic exposures. This time it was Vietnam where Dr. Viet reported elevated arsenic concentrations in groundwater in the Red River Delta. Since the conference, high levels in the Red River Delta in Cambodia have also been reported.

The seeds of the conference(s) were sown at a meeting of the SEGH Executive Board in December 1991. They agreed to form an Arsenic Task Force similar to the SEGH Lead in Soil Task Force that had been formed in the 1980s. It was clear that there was a growing controversy regarding the proposed changes in the US EPA MCL for arsenic in drinking water. This is the enforceable standard for drinking water. In addition to impacting on water utilities, the development of the standard would also have the possibility for significant economic impacts on the cleanup of superfund sites and on the electric power industry (because of arsenic in fly ash).

The Task Force was formed in 1992 and chaired by Willard Chappell. An international conference seemed to be an excellent way to begin to compile the data and to determine what needed to be done in this area. The First SEGH International Conference on Arsenic Exposure and Health Effects was held in New Orleans in 1993. This conference was successful in attracting the top arsenic researchers in the world. It was followed by the Workshop on Epidemiology and Physiologically-Based Pharmacokinetics that was held in Annapolis, MD in 1994. Perhaps the most significant outcome of the 1994 Workshop was

the realization that there are many arsenic hot spots in the world. Although not widely recognized by the scientific community at that time, significant public health problems existed in countries such as India, Thailand and China. The Second SEGH International Conference on Arsenic Exposure and Health Effects was held in 1995 in San Diego with a primary purpose being to highlight the global aspects of the problem and most of the impacted countries were represented. At that time, the biggest recognized problem area was in West Bengal, India where an estimated 30 million people are at risk from arsenic exposure in the ground water.

The Third SEGH International Conference on Arsenic Exposure and Health Effects (1998) was also held in San Diego and continued the theme of global impact of arsenic. In addition, two new countries with significant arsenic problems, Inner Mongolia and Bangladesh, were represented. The attendees were to learn that the Bangladesh problem could be larger than the one in West Bengal with a possible 80 million people (two-thirds of the population) at risk. This situation caught the attention of the media later in 1998 with the publication of a front-page article in the November 16, 1998 New York Times. The article was syndicated and published in newspapers around the world.

The SEGH Fourth International Conference on Arsenic Exposure and Health Effects continued the focus on the global impact of arsenic, and also featured sessions on mechanisms of cancer carcinogenesis, metabolism, and water treatment technology. We believe the 4th conference is the most dynamic conference to date and this resulting monograph represents the state-of-the-art in arsenic research on a worldwide basis. We believe that it will contribute to the solution of the many problems existing throughout the world and are very grateful to the authors for their diligence and fine work and to the sponsors for the support that made it possible.

We are also deeply appreciative of the fine efforts of Rosemary Wormington of the Environmental Sciences Program of the University of Colorado at Denver who put in long hours as conference coordinator. She kept the entire Conference going and, more than anyone else, is responsible for the success of this and the past conferences.

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Arsenic Exposure and Health Effects IV
W.R. Chappell, C.O. Abernathy and R.L. Calderon (Editors)
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Obituary: KURT J. IRGOLIC 1938—1999

It was a big shock when we heard of the fatal accident of our Department Head in the Austrian mountains on the 23th of July 1999.

BRIEF CURRICULUM VITAE

Kurt Irgolic was born on 28-9-1938 in Hartberg, a town in the southeast of Austria. He finished his education as primary school teacher in 1957. At that time the unemployment rate for school teachers was very high so he decided to study chemistry at the Karl-Franzens-University Graz, because he was always very interested in chemistry. The real reason for his decision to study chemistry was his mother because she preferred a chemistry student, who is sometimes at home to an unemployed primary school teacher who is home all the time (Kurt J. Irgolic June, 1999). He finished his Ph.D. at the Institute of Inorganic and Analytical Chemistry with Prof. Musil in January 1964, married his wife Gerlinde on the 1st of February and left Austria on the 22nd of February to do a Post Doc at the Department of Chemistry at the Texas A&M University in College Station, Texas, possibly assisted by the uncanny similarity of his East Styrian accent to a Texas drawl (Kurt J. Irgolic June, 1999). In 1966 he was appointed Assistant Professor, and was given the task of organizing a Freshman Chemistry course for about 2000 students a week. His daughter Birgit was born in September 1969. After six years he was promoted to Associate Professor. Another six years later he became full Professor of Chemistry. From 1972 through 1975 he was Research Coordinator at the Texas A&M University and was responsible for motivating scientists to write grant applications to bring in some money. Responding to the oil crisis the Texan government invested money for research in the field of energy resources and raw materials. As Associate Director of the Centre for Energy and Mineral Resources he had to distribute about 2 million US$ a year among the research groups at Texas A&M. From 1986 to 1989 he was chairman of the Inorganic Division in the Chemistry Department of Texas A&M.

After 25 years in Texas he came back to Graz in September 1989 as Professor for Analytical Chemistry at the Karl-Franzens-University. Immediately upon his return he became Chairman of the Office for International Affairs at the University. In this position he was responsible for student exchange programs as well as for supporting scientists from the University of Graz to attend international conferences. From 1996 to 1999 he was the Dean of the Natural Science Faculty.

CHAIR OF THE INSTITUTE OF ANALYTICAL CHEMISTRY KARL-FRANZENS-UNIVERSITY GRAZ

When Kurt Irgolic became Professor of Analytical Chemistry at the Karl-Franzens-University of Graz in September 1989, he found himself at the head of a deeply sleeping

department. The equipment was dominated by burettes and platinum crucibles. The most modern instrument was a HP UV/Visible spectrophotometer. With his experience from Texas A&M, he knew that good research in analytical chemistry is only possible with modern instruments. In his negotiations with the Science Minister he was very successful and obtained enough money to wake up the department and to modernize the equipment. In 1990 the department bought a Laser-Microprobe Mass Analyzer and an ICP–MS. Besides these two big instruments several smaller instruments like flame- and graphite furnace atomic absorption spectrometers and a HPLC-system were purchased. The improvement of the instrumental situation at the institute resulted in an increasing number of diploma and Ph.D. students and of course in an increase of scientific publications — although, before a manuscript could be submitted it had to go several rounds with Irgolic. After his corrections, our publications had a rejection rate close to zero. By the time he died, the department of Analytical Chemistry had become a modern, excellently equipped department with three ICP–MS instruments, several HPLC systems, a CE, various AAS spectrometers and all the necessary equipment for research in inorganic analytical chemistry. The money he brought in — whether from the government, research councils, or contracts with industry — was invested exclusively for purchasing equipment necessary for research. He did not allow money to be spent on things like furniture. In 1993 the Austrian government brought in a statutory reform of universities. This transferred quite a lot of responsibility from the government to the universities. Kurt Irgolic recognized the chance to amalgamate some small departments into a unified chemistry department. His Texas A&M experience told him that a bigger department would be much more flexible with respect to personal resources, use of instruments and equipment purchasing, and could also cut administrative costs. He invested many hours in convincing people from the Inorganic-, Organic-, Physical-, and Theoretical Chemistry to take this chance. It was a very sad moment when in December 1999 the Opening of the Institute of Chemistry (now about 150 people including Ph.D. students) took place without Kurt Irgolic. The foundation of the new big Institute of Chemistry was his baby and he would have been very proud at that moment.

RESEARCH

Kurt Irgolic had the conviction that science only works when it is interdisciplinary. His vision on science is manifested in the close cooperation with epidemiologists, geologists and biologists all over the world. The elements arsenic and selenium were definitely his dominant research topics. His profound knowledge on arsenic was the reason why he was a member of the Arsenic Task Force of the Society of Environmental Geochemistry and Health since 1993. Until a few years ago, knowledge of arsenic compounds in the terrestrial environment was restricted to the quite simple forms. In 1995 our research group, together with collaborators from Slovenia and Switzerland, identified for the first time arsenobetaine in mushrooms from Slovenia, an arsenic compound that was previously believed to exist only in the marine environment. In the following years almost all arsenic compounds detected earlier in the marine environment were also identified in the terrestrial environment. His work in the field of arsenic was recognized by the Japanese Society of Arsenic Scientists, which awarded him honourary membership in 1995. In collaboration with clinicians in Graz, investigations of trace elements in different human organs and body fluids was of big interest to him. He realized that all the elements in the human body are important in life. The so-called normal ranges, that can be found in the literature, are very wide and often based on old data determined with inadequate analytical methods. For this work, a second ICP–MS together with a digestion system was purchased in 1999 and installed in a clean room to obtain reliable results and to narrow the wide ‘normal range values’. A month before his death he initiated an alpine study with the aim to investigate the trace element status of people when they are hiking up to 2000 m

altitude. His fascination with the trace element status of human beings also led him to analyzing samples of Oetzi, the 5000 year old Man in the Ice found in Tyrol, and Juanita, a 500 year old female mummy, from Peru. For his efforts in Peru he got an honourary professorship of the Universidad Catolica de Santa Maria in Arequipa, Peru.

When he got a scientific manuscript for reviewing, and he got many, he took his job really seriously, which is nowadays not always a matter of course. It was not unknown that his suggestions and corrections filled more pages than the original manuscript. A detailed and clearly described experimental part in a manuscript was most important for him.

THE DEAN

When Kurt Irgolic became Dean of Natural Sciences at the Karl-Franzens-University Graz only a bare majority voted for him. The reason for this was obvious. During the time he had been chairman of the office for International Affairs he revolutionized our old traditional university in a way which was very difficult for some people. When scientists applied for travelling grants he did not make any difference between a full professor or a young assistant professor. All of them had the same chance to get money provided the publication list showed some recent entries. As Dean he had a budget of about 10 million US$ per year to distribute among the institutes of the faculty. He was strictly against the indiscriminate distribution of the money. He always supported successful scientists. All his efforts were to motivate the people and improve the reputation of our university. Egoistic thinking was not his style, he tried to be fair and always saw the whole system. This was sometimes difficult for our department because as Dean, he tried to avoid the accusation of treating his own department preferentially — thus we had to provide really good justification for buying new equipment. During his time as Dean he invested a lot of his time in helping to develop the University of Shkodra in Albania, a partner university of the Karl-Franzens-University of Graz. He organized used book transports from all his friends in the United States to Shkodra, he collected scientific equipment to build up a chemistry institute in there, and made several trips there to clean the old labs, to install electricity and water. One day, he was helping to pack a truck bringing books and used scientific equipment to Albania, and then turned up at a big faculty meeting in his blue overalls. For some people it was strange to have the Dean in this outfit at the faculty meeting. But that's how he was.

Some people respected his style but many could not be changed and it became inevitable that the he would lose the elections for a second term of office as Dean. On the day he lost the election, a month before his tragic accident, I saw him the first and only time in my life somewhat downcast.

STUDENTS

The relation of Kurt Irgolic to the students was ambivalent. One group, the motivated ones appreciated him, the others spread around that he was unfair. Indeed, many students failed his exams. The reason for that was definitely not him but the students themselves. I remember very well how disappointed he was when he corrected the exams and many students failed. He tried to find an answer why this happened. During his lectures he always tried to motivate the students to ask questions if something was unclear, but he could not break the big barrier which Austrian students feel when they talk to an university professor. He even organized monthly meetings in the Dean's office to discuss possible problems at the university. Again a thing unimaginable before him. During the summer holidays in Austria he organized several language courses for the students in Austria.

I personally met him at the first time as diploma student in 1990. In all his lectures I attended I could feel his enthusiasm for natural sciences and especially chemistry. After my diploma thesis I started Ph.D. with him in 1991. It was not always easy to get time with him to discuss the work. But even after a 12 hours working day at the Dean's office he was friendly, humorous and listened patiently to the scientific problems of his co-workers and students.

PRIVATE

Kurt Irgolic did not smoke and did not drink any alcohol, but he was tolerant. When people finished their exams and drank some champagne, a usual thing in Austria, he always joined the group and made some jokes but never tasted the champagne. Many people are smoking in the institute but he never said a word against it. One of his favourite sports was soccer. There was only one fixed date in his schedule, Wednesdays at 5 p.m. At that time he went home to Hartberg to do gymnastics and afterwards play soccer. His hobbies were hiking, looking for minerals and soccer. Unfortunately, his tight time schedule allowed him only a few days in the year to hike in the mountains. Then he walked alone in the Austrian alps. He quite often mentioned that if he had a choice he wanted to die in the mountains.

W. Goessler

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Arsenic: the 'King of Poisons' in Antiquity — a Possible Threat to Future Sustainability

Iain Thornton

ABSTRACT

The toxic nature of arsenic compounds has been recognised since the 15th century or earlier. There is also a long history both of arsenic being administered as a poison to commit murder and of adverse health effects in those working with arsenic and those exposed to arsenic containing products.

Present day concern is with less acute exposure. This paper notes some current research issues concerning (a) geochemical processes and pathways that influence the release of arsenic from natural geological sources into groundwaters, (b) possible impacts of arsenic contaminated irrigation waters on food quality and phytotoxicity, (c) the regulation of arsenic contaminated land in relation to potential health effects and (d) specific problems due to natural and mining sources of arsenic in river waters in Chile.

Agenda 21, one of the main outputs from the 1992 Earth Summit in Rio de Janeiro, committed Governments to a variety of social, economic and resource management issues within the general umbrella of sustainable development. How global arsenic problems can be addressed and tackled within these obligations has yet to be decided.

Keywords: arsenic; geochemistry; irrigation; contaminated land; sustainable development

INTRODUCTION

Arsenic compounds, in particular Orpiment (As_2S_3) and Realgar (AsS) were used as colouring agents and alloys in Greco–Roman times in ornamental painting and as cosmetics (Table 1). Arsenic bronzes and copper arsenic alloys were also used in antiquity, perhaps giving rise to the first recorded occupational disease, i.e., lameness in metalsmiths. Indeed it has been suggested that their unhealthiness stemmed mainly from the use of arsenical materials (Azcue and Nriagu, 1994).

Yellow orpiment, which closely resembled gold, was used for painting shields and lances and for writing in the fifteenth century, and at the same time its poisonous nature was recognised (Cennini, 1437, cited in Azcue and Nriagu, 1994).

More recently, in the late 19th century arsenic compounds were widely used as colouring agents, such as Paris Green and Kings Yellow, in wallpapers, wrapping paper and toys (Azcue and Nriagu, 1994). This use was in due course banned in the UK in 1880 because of toxic hazards.

Other main uses of arsenic in antiquity were pharmaceutical and medicinal, for example, as a remedy for asthma and coughs, and for a wide variety of problems in Indian medicine (Azcue and Nriagu, 1994). Fowler's solution (potassium arsenite), discovered in the late eighteenth century, was used widely as a remedy for a variety of illnesses for around 150 years (Frost, 1984).

In the 20th century, and indeed today, arsenic has been and still is widely used: (a) in agriculture, in pesticides, cotton defoliants, wood preservatives, as a growth promoter in pigs, in cattle and sheep dips, and in veterinary medicine for disease prevention; (b) in medicine, to treat for example sleeping sickness and syphilis; (c) in electronics and metallurgy and for other industrial uses including manufacture of glassware, catalysts and anti-fouling paints.

These uses have given rise to varying degrees of contamination in the surface environment and in some cases have increased levels of human exposure. Regulations controlling exposure in the workplace and defining permissible levels in environmental media have raised public awareness of possible hazards and led to reductions and even banning of certain applications. However, continuing use in developing countries is still a cause of concern.

However, it is arsenic's reputation as a poison, and indeed as the 'king of poisons' that has perhaps attracted most attention over the ages, and in a way provides the backdrop

TABLE 1

Arsenic uses through the ages

Antiquity	Arsenic bronzes Ornamental/painting Cosmetics Copper arsenic alloys
More recent to 19th century	Pharmaceutical Medicinal Colouring agents in toys, wallpapers and wrapping papers
20th century	Pesticides/insecticides Cotton defoliant Growth promoter in pigs Copper–chrome–arsenate wood preservative Wire alloys Electronics Glassware

to the current worldwide concern in the adverse health effects of long-term low level exposure, the subject of this and previous international conferences.

Agricola (1546) referred to two products, made by alchemists, one yellow and the other white, which he described as the most powerful poisons of the time, whether administered intentionally by those wishing to commit murder or due to accidental exposure by those working with arsenic compounds or using products painted or coated with arsenic pigments. The suggestion that arsenic poisoning was a cause of the death of Napoleon due to its presence in green pigments of wallpaper has been the subject of a lengthy debate (Jones, 1982). This theory would now seem to have been discounted with the further analysis of hair samples, and there is now strong evidence from autopsy findings that he died from cancer (Corso and Hindmarsh, 1996).

Some accidental mass poisonings have occurred, including that of 1900 when several thousand people were intoxicated by arsenic-containing beer in Manchester, UK (Reynolds, 1901), and in Japan in the 1950s where some 12,000 infants were fed dried milk containing pentavalent inorganic arsenic (Hamamoto, 1958) (both cited in Pershagen, 1986).

Whether historically the recognition of the toxicity was a result of observation of acute effects of poisoning or chronic effects such as we recognise today is not certain. Perhaps adverse effects on health were widespread but largely unrecognised.

My presentation in the 3rd Symposium in 1998 (Thornton, 1999) was based on a request to attempt to gaze into a crystal ball and attempt to predict where arsenic research would progress in the future. On that occasion I focussed on three main aspects: (a) the rapidly increasing knowledge of sources and locations of arsenic enrichment in the environment and the worldwide significance to human health; (b) the wide-ranging scientific issues that have and will continue to emerge, including the need to identify and understand the importance of the chemical and mineral species of arsenic in the environment, the geochemical processes controlling pathways and their implications to human exposure, and the nature of the dose–response curve and its influence on toxicity and carcinogenicity; (c) actions necessary to reduce exposure based on further development of the regulatory process and applications of methods of remediation where water and land contamination had already taken place.

This present paper will briefly review some of the research progress over the past two years and will introduce some of the current issues requiring attention.

GEOCHEMICAL PROCESSES AND PATHWAYS

The scientific debate regarding the nature of natural arsenic sources and their potential solubility in geological beds in West Bengal, India and Bangladesh has been active over the past two years (Acharyya et al., 1999; Chowdhury et al., 1999; Nickson et al., 1999). Whether arsenic is released from geological strata under oxidising or reducing conditions is discussed further in subsequent papers by Smedley et al., Chowdhury et al., and McArthur et al. (this volume). The practical implications of this debate are of immense importance to those responsible for future decisions on handling the calamitous situation involving the exposure of several million local population to dangerously high concentrations of arsenic in their drinking water.

A further issue has been raised by K.C. Sahu (pers. commun., 1999), an eminent Indian geologist who considers that the sources of this arsenic in the Ganges Basin are not from natural geological beds but due to man's activities, including the burning of coal, the use of fertilisers and pesticides. This provides an interesting and yet unproven view.

Under the very different scenario in the USA, the Safe Drinking Water Act which requires USEPA to review current drinking waters for arsenic has led to the examination of water quality data from some 18,850 locations selected from the US Geological Survey National Water Information System (NWIS) (Focazio et al., 1999; Welch et al., 2000). Of the

groundwaters analysed, about 10% exceeded 10 μg/l As and 1% of public water supply systems exceeded 50 μg/l. Raised levels were more frequently found in the western US than in the east and were more widespread than previously recognised. The most common source was arsenic released from iron oxides as a result of geochemical conditions, including the reaction of iron oxide with either natural or anthropogenic sources of organic carbon. Other sources are sulphide minerals and geothermal waters. Increased arsenic concentration due to evaporation is important in closed hydrologic basins of the semiarid western United States where evaporation exceeds precipitation (Welch et al., 2000).

IRRIGATION

Very little is as yet known as to the potential impacts of using contaminated well waters in India, Bangladesh and elsewhere for the irrigation of food crops (W.R. Chappell, personal commun., 2000). It has been noted that within one area of 200 km^2 in the Ganges Basin, irrigation water from 3200 tube wells has deposited over 6 tonnes of arsenic on agricultural land. This gives some indication of the massive scale of the situation in West Bengal, India and Bangladesh (D. Chakraborti, personal commun., 1999). There are two possible impacts. Firstly, the effect on food quality in which arsenic from water is accumulated in the food crop and provides an additional source of human exposure. The rice plant in particular has been shown to take up much larger amounts of arsenic than other food crops, perhaps due to the density of the soils in which rice is grown. It has been suggested that flooded paddy soils produce an anaerobic environment that mobilises arsenic, promoting greater uptake by plants (Schoof et al., 1999). Secondly, and perhaps more important, is the problem of phyto-toxicity as the rice plant has already shown to be susceptible to adverse effects from arsenic (Huang, 1994).

Although it has not yet been possible to confirm increasing concentrations of arsenic in paddy soils in the Indian subcontinent, it has been strongly recommended that field-based research should now be focussed in priority areas to establish the extent of the problem (W.R. Chappell, personal commun., 2000).

In the Rajarampur area of Bangladesh, it has been suggested that raised arsenic concentrations in surface waters (ranging up to 176 μg/l) are derived from contaminated groundwater used for irrigation (Islam et al., 2000).

Research has already been undertaken in northwest Chile where arsenic-contaminated irrigation waters have given rise to increases in the arsenic content of vegetable crops. These vegetables are consumed by the indigenous population (Sancha and Marchetti, 2000).

CONTAMINATED LAND

While the main emphasis in this Symposium is on arsenic in waters as a source of human exposure, as indicated previously, arsenic arising from past mining and smelting activities (Thornton and Farago, 1997) and from the use of pesticides and defoliants may give rise to excessive amounts of arsenic in surface soils, both agricultural and urban.

In the UK, a new 'Contaminated Land Regime' was introduced in April 2000 as Part IIA of the Environmental Protection Act. The main emphasis of this regime concerns the protection of human health, though wider environmental concerns consider the protection of the ecosystem. Some 260 local Government Authorities were required within 18 months to draw up registers of contaminated sites, with a view to regulatory planning for future development using a fit-for-purpose approach. Sites under current usage for housing, agriculture etc., are also included. There are obvious implications to the future value of property and land which may, in due course, lead to what is colloquially termed 'planning blight'.

TABLE 2

Current definition of contaminated land in the UK

Land contaminated to a degree that:
(a) significant harm is being caused;
(b) there is a significant possibility of such harm being caused;
(c) pollution of controlled waters is being caused; or
(d) pollution of controlled waters is likely to be caused.

The present definition of contaminated land is somewhat vague as shown in Table 2.

The regime embraces the concept of pollutant linkage from land to sensitive receptors. The process in due course will be driven by guideline values derived from a risk assessment model (CLEA). These will be based on total arsenic concentrations in soils, and will not take into account arsenic speciation or changes in speciation with time due to ageing/transformation. Thus, no account will be taken of arsenic bioavailability or bioaccessibility.

The implementation of the Contaminated Land Regime will be based on the application of the 'Precautionary Principle' implying that if scientific evidence and proof of hazard are incomplete, one should stray on the side of caution both to protect the population and the ecosystem.

There is at present very little evidence linking contaminated land and impacts on human health, and for arsenic any relationships proposed would seem to be empirical rather than causal.

Isolated health incidents have been attributed to arsenic poisoning due to ingestion/inhalation of dusts from mine wastes in southwest England; these include skin disorders and physical fatigue and mental tiredness (Hamilton, 2000). However, as reported previously, although raised urinary levels have been reported in individuals living in the vicinity of old mine wastes (Kavanagh et al., 1998), no adverse health effects were associated with these. An ecological study which examined bladder cancer rates in relation to levels of environmental arsenic in parts of southwest England showed no significant relationship between the two (Leonardi et al., 1995).

Current research funded by the European Commission and involving Imperial College, London, Charles University, Prague and others focuses on possible adverse health effects of arsenic emitted from a large coal-burning power station in the Nitra Valley in Slovakia. Coal containing up to 1000 mg/kg arsenic has been burnt in the past. Even at this time cleaner coal that is used still contains up to 400 mg/kg As. Research comprises a Case Control Study of skin cancers and Ecological Studies of skin, lung and bladder cancers with appropriate environmental measurements of arsenic concentrations in housedusts, garden soils and amenity areas at various distances around the plant (see poster presentation, Keegan, Farago and Thornton).

SURFACE WATER CONTAMINATION IN CHILE

The River Loa and its tributaries, flowing from the Andes Mountains and their foothills to the sea to the north of Antofagusta is contaminated by arsenic both from natural (volcanic and geothermal) sources in the headwaters, and from effluents arising from the mining and smelting of copper ores at Chuquinamata (responsible for some 10% of the economy of Chile). Current research funded by the European Commission involving partners in Sardinia, France, UK and Latin America aims to (a) apportion the magnitude of these natural and man-made sources of arsenic, (b) assess the importance of chemical and physical factors influencing the dispersion of arsenic in this river system, and (c)

evaluate possible remediation strategies. Problems arising from human consumption of this arsenic-rich water have previously been documented (Hopenhayn-Rich et al., 1999) and impacts of using this water for the irrigation of vegetable crops are illustrated in the poster presentation of Sancha.

Occasional flooding within the river system, as in the year 2000, has resulted in the dispersion of arsenic-rich sediments into inshore waters with a major effect on the commercial value of the fish (some 200,000 tonnes per year) previously processed and exported as fish meal to countries of the EU, but now banned because of the contamination with arsenic. This loss has been valued at around US$ 80 million per year (H. Alonso, personal commun., 1999).

ARSENIC AND SUSTAINABLE DEVELOPMENT

It is difficult to obtain reliable information on the actual impacts of arsenic contamination, both natural and man-made, worldwide on national economies, human health and the overall environment. There are obvious implications to current and future agricultural production, riverine and inshore fisheries, urban development and planning, and overall land use. One of the main outputs from the 1992 Earth Summit in Rio de Janeiro was the acceptance of Agenda 21 by the majority of Governments, who are and will continue to be committed to the implementation of its various chapters. Some of these are listed in Table 3.

It is not certain to what extent the implication of Agenda 21 will be fed into the current global arsenic debate. It would seem the responsibility of individual national governments to promote and protect the well-being, future development and health of their populations, be it in the United States, India or elsewhere.

It is also the responsibility of those in more affluent countries to assist where problems exist. It would now seem appropriate to examine and assess how these responsibilities are being handled in what continues to be a rapidly developing and complex scientific and political arena.

TABLE 3

Earth summit: Rio de Janeiro 1992, Agenda 21

Chapters relevant to the arsenic debate in relation to sustainable development:	
Section I, Social and Economic Dimensions	
• Chapter 3	Combating poverty
• Chapter 4	Changing consumption patterns
• Chapter 5	Demographic dynamics and sustainability
• Chapter 6	Protecting and promoting human health
• Chapter 7	Promoting sustainable human settlement development – sustainable land-use, planning and management – water, sanitation, drainage
• Chapter 8	Integrating environment and development in decision making
Section II, Conservation and Management of Resources for Development	
• Chapter 18	Protection of the quality and supply of freshwater resources: application of integrated approaches to the development, management and use of water resources
D.	Drinking water supply and sanitation
E.	Water and sustainable urban development
F.	Water for sustainable food production and urban development
Section IV, Means of Implementation	
• Chapter 34	Transfer of environmentally sound technology, co-operation and capacity building

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International Perspective on Naturally Occurring Arsenic Problems in Groundwater

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ABSTRACT

Arsenic can cause severe problems in a number of aquifers as a result of its relatively high solubility in many natural groundwaters and its toxicity. High concentrations can be found under both oxidising and reducing conditions. Most problems are found in young aquifers having slow rates of groundwater flow such that aquifer flushing, and hence arsenic removal, has been restricted. Affected oxidising aquifers are often in arid or semiarid regions where recharge is low. In these, pH and salinity are often high, arsenic is dominated by As(V) and may be associated with high concentrations of other anions and oxyanions (F, HCO_3, V, Mo, Se, U, B, Be). Arsenic mobilisation is most likely a result of desorption from oxide minerals under the high-pH conditions. Examples include the Quaternary sedimentary and volcanic aquifers of central Argentina, northern Chile, north-central Mexico and parts of southwest USA. Affected reducing aquifers are typified by groundwaters with high Fe, Mn and NH_4 concentrations and often show evidence of sulphate reduction. Arsenic(III) typically forms a high proportion of the total arsenic present. Mobilisation of arsenic is likely to be dominantly by reductive desorption from, and dissolution of, iron-oxide minerals. Examples include the Quaternary aquifers of Bangladesh and West Bengal, Inner Mongolia, Taiwan, Vietnam, Hungary and Romania as well as parts of the western USA. Examples of arsenic occurrence are described in groundwaters from the strongly reducing alluvial/deltaic aquifers of Bangladesh and lacustrine/alluvial aquifers of the Huhhot Basin, Inner Mongolia, as well as from the oxidising loess aquifer of northern La Pampa, Argentina.

Keywords: arsenic; groundwater; geochemistry; Argentina; Bangladesh; China

INTRODUCTION

An increasing number of cases of arsenic contamination of water and soils have been reported recently in various parts of the world (Fig. 1). Many are located in areas of sulphide mineralisation where oxidation of sulphide minerals, particularly exacerbated by mining activity, has led to release of arsenic into the surrounding environment (e.g., Welch et al., 1988; Ficklin and Callender, 1989; Azcue and Nriagu, 1995; Grimes et al., 1995; Smedley et al., 1996; Williams et al., 1996; Boyle et al., 1998). Other cases of contamination are associated with geothermal activity, with notable documented occurrences from New Zealand, Chile, Argentina, the Caribbean region and western USA (e.g., Ellis and Mahon, 1979; Welch et al., 1988; Criaud and Fouillac, 1989). These two types of environments can have serious cases of arsenic contamination, with often high concentrations (in the milligram-per-litre range) in water. However, the contamination is typically localised to the vicinity of the contaminant sources as a result of the strong arsenic-adsorbing capacity of soils and sediments. They are also often easily recognised and have often been closely monitored.

More insidious is the problem of arsenic contamination of groundwaters in some major aquifers which can be extensive and can affect large numbers of people if the groundwaters are relied upon for potable supply. Contamination of groundwaters in aquifers may go unnoticed for long periods if no routine testing for arsenic takes place. Cases of natural contamination of groundwaters with arsenic have been documented from parts of Taiwan, northern China, India (West Bengal), Bangladesh, Hungary, Romania, Vietnam, Argentina, Chile, Mexico and western USA. (Fig. 1; Tseng et al., 1968; Welch et al., 1988, 2000; Nicolli et al., 1989; Del Razo et al., 1990; Luo et al., 1997; Berg et al., 2001; Gurzau and Gurzau, 2001; Kinniburgh and Smedley, 2001). The extent and levels of contamination vary significantly in these areas, but many have arsenic-bearing groundwaters which extend over several tens to hundreds of kilometres.

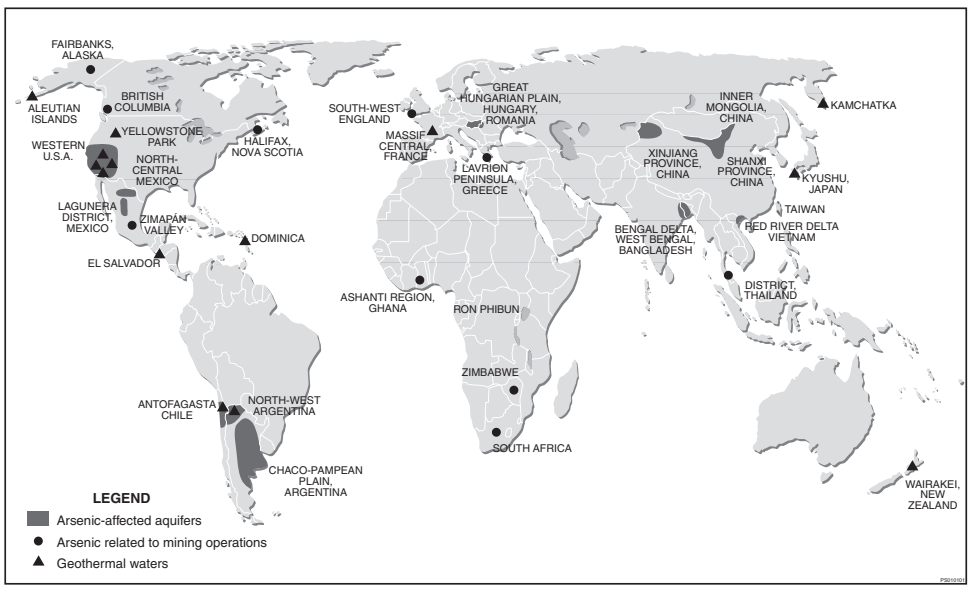

Fig. 1. World map of documented arsenic problems in groundwater and the environment. Cases include mining-related and geothermal occurrences.

Although some differences occur in groundwater chemistry between each affected region or aquifer, a number of common features exist that allow generalisations to be made about the key controls on arsenic mobilisation in water and the types of aquifer that are most at risk of contamination.

This paper describes the results of hydrogeochemical investigations carried out in aquifers from three countries with recognised and serious arsenic problems: Bangladesh, Inner Mongolia (China) and Argentina. The study areas are the upazilas (administrative units) of Lakshmipur, Faridpur and Chapai Nawabganj in Bangladesh, the Huhhot Basin in Inner Mongolia and part of the province of La Pampa in central Argentina. In each region, the principal water-chemistry and aquifer characteristics are described and comparisons are made with other arsenic-affected aquifers worldwide. The paper highlights some of the main geochemical controls on arsenic mobilisation and assesses the characteristics of aquifers likely to be at risk.

METHODOLOGY

Samples of pumped groundwater were collected from each study area over the period 1996–1999. Samples were mostly collected from tubewells after initial purging, although some from the Huhhot Basin and La Pampa were collected by bailing from open wells. In each study area, samples were collected with a broadly even spatial coverage and, in most cases, were selected without prior knowledge of their arsenic concentration. On-site measurements included water temperature, pH, redox potential (Eh), dissolved oxygen, specific electrical conductance (SEC, 25°C) and alkalinity (given as HCO_3). Where possible, well depths and water levels were also recorded.

Water samples were also collected for subsequent laboratory analysis. Samples for major- and trace-element analysis were filtered (0.45 μm or less) into acid-washed polyethylene bottles. Aliquots for analysis of major cations, SO_4 and trace elements were acidified and analysed by ICP–AES and ICP–MS. Total As (As_T) and As(III) were measured by either ICP–AES or AFS, each with hydride generation. Analysis of Cl, nitrogen species and F was carried out by colorimetry on filtered but unacidified aliquots. Carbon-13 was analysed by mass spectrometry. Further details of the sampling and analytical techniques are given in Kinniburgh and Smedley (2001) and Smedley et al. (2001).

RESULTS

Bangladesh

Arsenic problems in Bangladesh occur in groundwaters from Holocene alluvial and deltaic aquifers associated with the Ganges, Brahmaputra and Meghna river systems. These cover a major proportion of the Bangladesh land area. It has been found that groundwaters from deep aquifers (generally >150 m depth) underlying the shallow Holocene sediments, as well as groundwaters from upfaulted older sedimentary formations (e.g., Pleistocene sediments of north-central Bangladesh), have low arsenic concentrations, almost all being <10 μg l^{-1} (e.g., Kinniburgh and Smedley, 2001). A recent national survey of some 3500 groundwater samples collected on a randomised basis showed 46% exceeding the WHO guideline value of 10 μg l^{-1} and 27% exceeding the national standard of 50 μg l^{-1} (Kinniburgh and Smedley, 2001). Although the spatial variability of arsenic concentrations is high, the worst-affected parts of the Holocene aquifers are in the south and southeastern parts of the country, in the low-lying parts of the Bengal delta (Fig. 2). Groundwater from Holocene sediments in northern Bangladesh typically have low arsenic concentrations (<10 μg l^{-1}; Kinniburgh and Smedley, 2001).

Detailed hydrogeochemical surveys have been carried out in three study areas of Bangladesh: the upazilas of Chapai Nawabganj, Faridpur and Lakshmipur (Fig. 2). Chapai

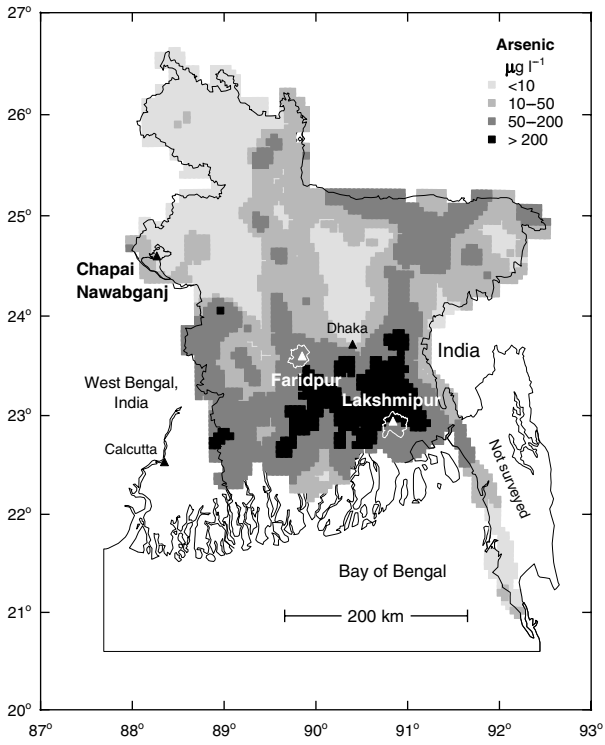

Fig. 2. Map of Bangladesh showing the location of the three study areas (upazilas) and the distribution of smoothed (kriged) As concentrations from a national survey of groundwaters (Kinniburgh and Smedley, 2001).

Nawabganj lies in northwest Bangladesh, close to the Indian border. Holocene sediments associated with the Ganges floodplain crop out in the western part of the upazila (the northeastern part having outcropping older Pleistocene sediments; Kinniburgh and Smedley, 2001). Faridpur lies below the confluence of the Ganges and Brahmaputra rivers and outcropping sediments are Holocene sands and silts of both alluvial and deltaic origin. Lakshmipur upazila is situated in the coastal belt of southeast Bangladesh, close to the present course of the Meghna River and downstream of its confluence with the Ganges and Brahmaputra rivers. Surface geology of Lakshmipur comprises Holocene alluvial silt and clay and tidal deltaic deposits, as well as a unit of Holocene sediments more oxidised and consolidated (the 'Chandina Alluvium'; Alam et al., 1990) than those characteristic of the active floodplain in the south of the upazila.

A summary of groundwater chemistry in the Holocene aquifers from each of the study areas is given in Table 1. Tubewell depths were <150 m in Lakshmipur, <100 m in Faridpur and <50 m in Chapai Nawabganj. The groundwaters are overwhelmingly reducing (low redox potentials and low or no dissolved oxygen) with often high concentrations of dissolved Fe, Mn and NH_4-N (Table 1). Arsenic concentrations are variable but often high. Of the three areas, the highest average concentrations and greatest proportion in excess of 50 $\mu g\ l^{-1}$ are found in Lakshmipur. Median arsenic concentrations in groundwaters from Lakshmipur, Faridpur and Chapai Nawabganj are respectively 89 $\mu g\ l^{-1}$, 39 $\mu g\ l^{-1}$ and 3.9 $\mu g\ l^{-1}$ (mean values, respectively, 159 $\mu g\ l^{-1}$, 102 $\mu g\ l^{-1}$ and <3 $\mu g\ l^{-1}$).

Analyses of groundwater from open dug wells show that these are typically more oxidising as a result of localised oxidation in the well vicinity or lack of reduction in the shallowest parts of the aquifers close to the water table. Groundwater from dug wells

TABLE 1

Summary of groundwater quality in aquifers from three study areas in Bangladesh

	Units	Lakshmipur shallow groundwater (<150 m)				Faridpur shallow groundwater (<100 m)				Chapai Nawabganj shallow groundwater (<50 m)			
		Min.	Max.	Median	*n*	Min.	Max.	Median	*n*	Min.	Max.	Median	*n*
Depth	m	4	36	11	59	14	88	29	59	6	41	29	67
Temp	°C	24.3	27.4	25.9	59	25.4	28.1	26.2	59	25.7	28.8	27	69
pH		6.66	7.59	7.18	59	6.38	7.35	6.94	59	5.9	7.32	6.99	65
Eh	mV	−105	160	82	59	18	309	101	59	−102	341	60	68
DO	mg l^{-1}	<0.1	1.4	<0.1	59	<0.1	1.4	<0.1	59	<0.1	0.9	<0.1	69
SEC	μS cm^{-1}	384	4640	886	59	344	1400	749	59	331	1370	726	69
Ca	mg l^{-1}	16.6	186	47.9	59	34.8	190	101	59	36.6	218	104	69
Mg	mg l^{-1}	13.9	154	42.6	59	8.9	59.5	31	59	9.0	49.2	24.6	69
Na	mg l^{-1}	7.9	1090	79.2	59	5.1	175	18.6	59	5.2	95.1	18.4	69
K	mg l^{-1}	3.5	41.3	11	59	2.6	8.7	4.7	59	0.5	18.7	4	69
HCO_3	mg l^{-1}	262	1140	463	59	200	848	536	59	199	826	464	69
Cl	mg l^{-1}	4.9	1550	67.7	59	1.8	127	9.3	59	1.0	70	6.9	68
SO_4	mg l^{-1}	<0.2	265	0.7	59	<0.2	64	<0.2	59	<0.2	115	7.4	69
NO_3-N	mg l^{-1}	<0.5	29.4	<0.5	59	<0.3	4.9	<0.3	59	<0.3	12.6	<0.5	69
NO_2-N	μg l^{-1}	<4	6210	<4	59	<4	4800	<4	59	<4	910	<4	69
NH_4-N	mg l^{-1}	<0.01	17.8	1.84	59	<0.06	17.6	1.02	59	<0.01	2.0	0.02	69
DOC	mg l^{-1}	0.4	14	3.1	57	0.1	13	1.9	48	0.2	9.4	1.3	68
Si	mg l^{-1}	7	25.1	14.5	59	6.3	41.9	16	59	7.4	22.3	14.8	69
P_T	mg l^{-1}	<0.2	3.4	0.68	59	<0.2	5.0	1.5	59	<0.2	1.14	<0.2	69
As(III)	μg l^{-1}	<1	501	57	59	<6	990	9.2	59	<1	2208	1	69
As_T	μg l^{-1}	<1	986	89	59	<6	1460	39	59	<1	2342	3.9	69
Fe_T	mg l^{-1}	0.122	24.8	1.71	59	0.052	19.6	5.57	59	0.021	9.79	0.455	69
Mn	mg l^{-1}	0.121	3.83	0.577	59	0.041	4.23	0.48	59	0.02	4.36	0.76	69
B	μg l^{-1}	39	818	156	59	10	105	32	59	10	102	25	69
F	μg l^{-1}	80	450	230	59	20	400	110	59	40	1280	160	69
V	μg l^{-1}	<0.2	2.78	<0.2	59	<0.2	1.77	<0.2	59	<0.2	2.55	<0.2	69
Cu	μg l^{-1}	<1	3	<1	59	<1	7	<1	59	<1	2	<1	69
Mo	μg l^{-1}	<0.1	20	2.95	59	<0.1	20.46	1.34	59	0.13	9.77	0.82	69
U	μg l^{-1}	<0.01	17.3	0.26	59	<0.01	29.6	0.013	59	<0.01	31.7	1.6	69
$\delta^{13}C$	‰	−23.6	−7.1	−17.4	56	−18.3	0.6	−12.9	21	−18.1	−6.8	−12.2	64

Temp = temperature; Eh = redox potential; DO = dissolved oxygen; SEC = specific electrical conductance; DOC = dissolved organic carbon.

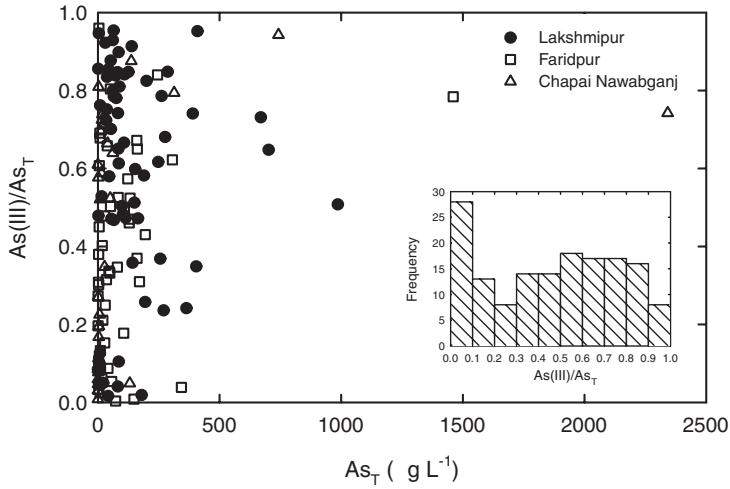

Fig. 3. Variation of As(III)/As$_T$ with As$_T$ in groundwaters from Bangladesh. Arsenic(III) forms a variable proportion of the total As present, but is typically high in high-As waters.

generally has low As$_T$ concentrations of close to, or less than, 10 μg l^{-1} (Kinniburgh and Smedley, 2001).

Under the reducing conditions found in the majority of the Bangladesh groundwaters, As(III) is found to compose a substantial proportion of the total dissolved arsenic concentration (Fig. 3). As(III)/As$_T$ ratios in each area are variable (<0.1 to >0.9) but are typically around 0.6 (i.e., As(III) is around 60% of the As$_T$) where dissolved As$_T$ concentrations are high. This suggests that reduction of As(V) to As(III) could be an important control on the release of As into solution. The groundwaters have high concentrations of dissolved Fe (up to 25 mg l^{-1}) and Mn (up to 4.4 mg l^{-1}) and also often high P (up to 5 mg l^{-1}; Table 1). Many contain dissolved inorganic carbon with strongly depleted values of $\delta^{13}C$ (often less than −20‰), a feature which is often taken to be related to oxidation of organic matter (e.g., Clark and Fritz, 1997). Oxidation of organic matter is believed to have been a key factor in the generation of the strongly reducing conditions in the Bangladesh aquifers and is a major cause of the high alkalinity values (up to 1140 mg l^{-1}) which are characteristic of the Bangladesh groundwaters.

Concentrations of SO_4 in the groundwaters are generally low, often around 1 mg l^{-1} or less (Table 1), a finding which suggests that some SO_4 reduction has taken place. Fig. 4 shows that a broad inverse correlation exists between As$_T$ and SO_4 in each of the study areas. The highest As concentrations occur where SO_4 reduction has been most pronounced. Arsenic release therefore appears to have occurred under the most reducing conditions.

Although the long-term effects of abstraction and agricultural practices on groundwater chemistry in Bangladesh are not clearly established, the release of arsenic into solution is considered to have been fundamentally a natural process that has occurred as a result of the evolution of the aquifers towards strongly reducing conditions. Such conditions are likely to have been produced by the rapid reaction of freshly buried organic matter.

Huhhot Basin, Inner Mongolia

The Huhhot Basin lies on the margins of the Yellow River Plain in Inner Mongolia and accommodates the capital city of Huhhot. The basin has an area of around 4800 km^2 and

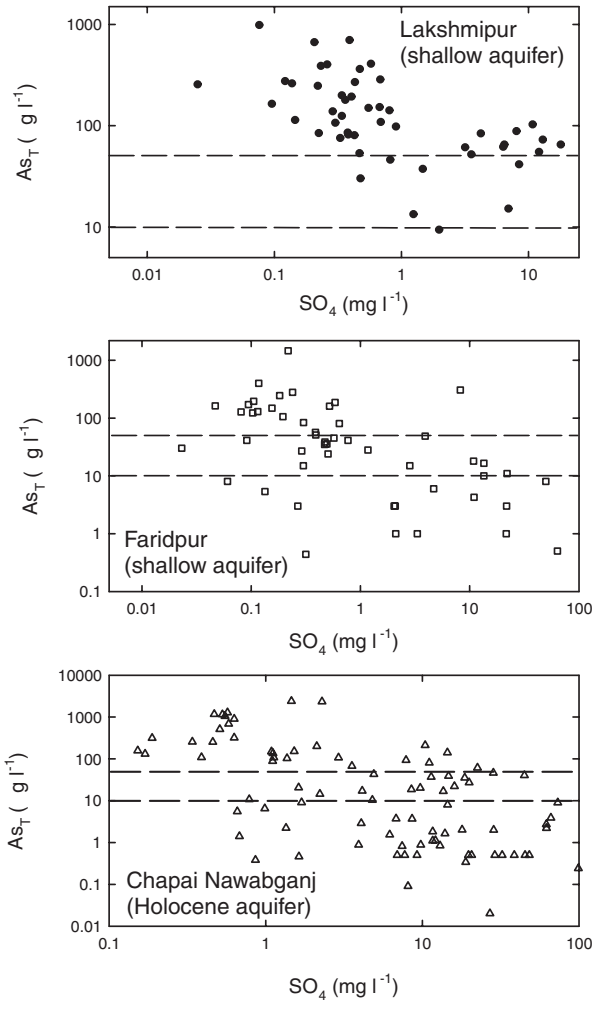

Fig. 4. Variation of As_T with SO_4 in groundwaters from the three studied areas in Bangladesh.

is bounded on three sides by mountains of the Da Qing and Man Han ranges (altitude around 2000 m; Fig. 5). The basin is infilled with a thick sequence of sediments, the principal aquifers being lacustrine and alluvial sediments of Holocene age. Basin margins have a predominance of coarse, principally arenaceous, sediments formed as alluvial-fan deposits. In the lower part of the basin, fine sediments predominate from a former lake, which occupied the basin until ca. 8000 years ago (Smedley et al., 2000).

The region experiences an arid climate (annual rainfall around 440 mm). The Black River, the main drainage channel, is an ephemeral river that dries up during the summer months due to high evaporation and irrigation losses. Rates of groundwater recharge are low (ca. 70 mm; J. Li, pers. commun., 1998), drainage to the Yellow River catchment is poor and groundwater flow rates are slow, particularly in the low-lying areas, with generation of an effectively closed basin. Both high seasonal evaporation and high irrigation losses have led to a degree of salinisation of the shallow aquifers.

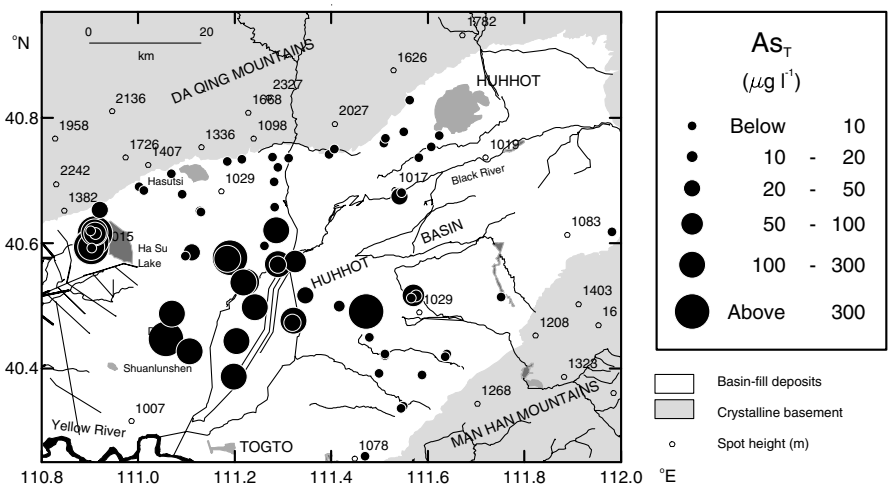

Fig. 5. Regional distribution of As_T concentrations in groundwaters from the Huhhot Basin, Inner Mongolia. High concentrations are restricted to anaerobic groundwaters in the central parts of the basin.

Groundwater abstraction is mostly from two depth ranges: <30 m and >100 m. The groundwaters from the two ranges show some distinctions, notably in salinity and concentrations of dissolved F and dissolved organic carbon. Higher salinity of some of the shallow groundwaters is related to evaporation, often exacerbated by irrigation. This has also had an influence on increased F concentrations, which have been measured at up to 6.8 mg l^{-1} (Table 2). Deep groundwaters have often high concentrations of dissolved organic carbon (up to 31 mg l^{-1}; Table 2) and humic acid (64 mg l^{-1}; Z.-d. Luo, unpublished data). Many of the deep groundwaters are artesian. Despite these differences between shallow and deep groundwaters, some similarities in chemical composition exist and hence summary statistics given in Table 2 have not distinguished between the two ranges.

Groundwaters from the Huhhot Basin display a clear redox gradient as they flow downhill from the foothills at the basin margins towards the lowest parts of the basin. Groundwaters at the basin margins are aerobic (containing dissolved oxygen and nitrate and with redox potentials up to 426 mV). In the low-lying central part of the basin, groundwaters become anaerobic. In these, concentrations of Fe reach up to 4.5 mg l^{-1}, Mn up to 1.3 mg l^{-1} and NH_4-N up to 18 mg l^{-1} (Table 2). As with Bangladesh, alkalinity values of many groundwaters are high (HCO_3 up to 1150 mg l^{-1}), as are concentrations of dissolved P (up to 3.1 mg l^{-1}; Table 2).

As a result of the large redox changes, concentrations of dissolved As_T also vary considerably from <1 μg l^{-1} to 1480 μg l^{-1} (median concentration 5.4 μg l^{-1}; mean 84.8 μg l^{-1}; Table 2). The high concentrations are restricted to the anaerobic groundwaters in the central parts of the basin, covering an area of some 2500 km^2 (Fig. 5). In the groundwaters where As occurs in solution, As(III) is found to be the main dissolved species. As(III)/As_T ratios are usually in the range 0.7 to 0.9 (Fig. 6).

Fig. 7 shows that concentrations of dissolved SO_4 are generally much lower in the anaerobic groundwaters in the central parts of the basin. This suggests that SO_4 reduction has been an important process and is indicative of strongly reducing conditions in the parts of the aquifers where As mobilisation in groundwater has occurred. The hydrogeochemical situation is therefore broadly analogous to that of Bangladesh.

TABLE 2

Summary of groundwater quality in aquifers from the Huhhot Basin, Inner Mongolia

	Units	Min.	Max.	Median	*n*
Well depth	m	<10	400	40	73
Temperature	°C	5.2	16.0	9.8	72
DO	mg l^{-1}	<0.1	9.8	0.4	72
Eh	mV	<2	426	154	72
SEC	μS cm^{-1}	463	4350	841	73
pH		7.00	8.56	7.78	73
Ca	mg l^{-1}	4.2	190	50.1	73
Mg	mg l^{-1}	3.5	192	35.2	73
Na	mg l^{-1}	10.6	835	104	73
K	mg l^{-1}	0.04	90.6	1.9	73
HCO_3	mg l^{-1}	182	1150	389	72
Cl	mg l^{-1}	6.1	919	74.3	73
SO_4	mg l^{-1}	<0.2	1007	34.6	73
NO_3-N	mg l^{-1}	<0.01	34.3	1.13	73
NO_2-N	μg l^{-1}	<3	3300	6	73
NH_4-N	mg l^{-1}	<0.01	18.0	0.02	71
DOC	mg l^{-1}	0.3	30.6	3.6	72
Si	mg l^{-1}	2.5	16.9	6.1	73
P_T	mg l^{-1}	<0.05	3.1	0.18	73
B	mg l^{-1}	<0.03	1.98	0.17	73
Fe_T	mg l^{-1}	<0.01	4.51	0.07	73
Mn	μg l^{-1}	<1	1289	26	73
Se	μg l^{-1}	<0.2	5.4	<0.5	73
As(III)	μg l^{-1}	<0.9	1290	5.2	73
As_T	μg l^{-1}	<1.0	1484	5.4	73
F	mg l^{-1}	0.13	6.78	0.53	73
V	μg l^{-1}	<0.2	10.3	0.9	73
Cu	μg l^{-1}	<1	2.5	<1	73
Mo	μg l^{-1}	<0.1	62.6	1.9	73
U	μg l^{-1}	<0.01	52.8	1.24	73
$\delta^{13}C$	‰	−0.2	−20.5	−11.0	40

Abbreviations as for Table 1.

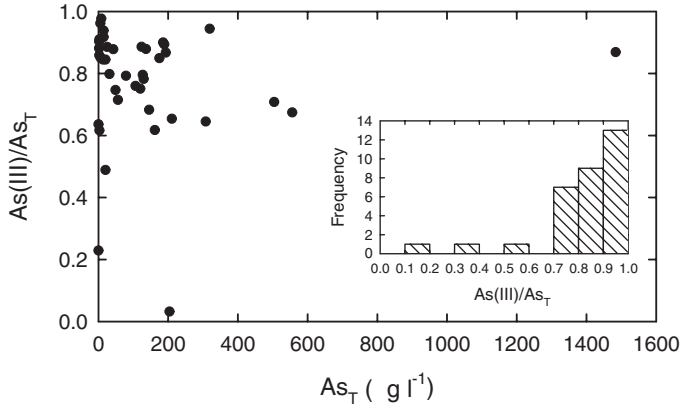

Fig. 6. Variation of As(III)/As_T with As_T in groundwaters from the Huhhot Basin. Arsenic(III) is usually a major component of the total dissolved As concentration.

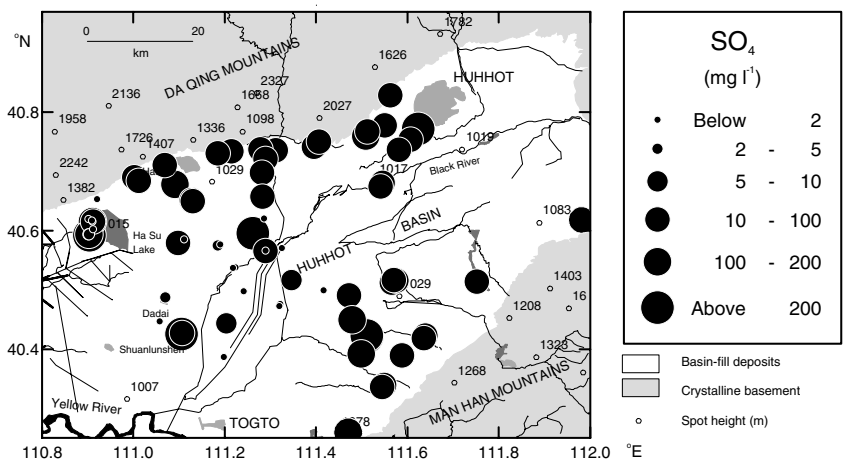

Fig. 7. Regional distribution of SO_4 in groundwaters from the Huhhot Basin.

One notable difference from the Bangladesh situation is in the chemistry of dug-well waters, a limited number of which were collected from the high-As area of the basin. Concentrations of As were found to be high in some (range $<$ 1–556 μg l^{-1}; $n = 4$) and some have maintained an overall reducing condition, despite being open to atmosphere. The waters with high As concentrations contain relatively high concentrations of dissolved organic carbon (up to 10 mg l^{-1}). This, together with solid forms of fresh organic carbon, may be responsible for maintaining the reducing conditions. The groundwaters from the arsenic-bearing dug wells are also thought to be dominated by upflowing discharging groundwater in the low-lying part of the basin, rather than by direct recharge. The dug-well waters also have unusually high SO_4 concentrations (34–254 mg l^{-1}; $n = 4$). The origin of the SO_4 is not clear but may be linked to some localised oxidation of sulphide minerals at the shallowest levels of the aquifer.

La Pampa, Argentina

The province of La Pampa lies in the semiarid region of central Argentina. The province forms part of the vast Chaco–Pampean Plain which extends to the Andean foothills in the west, the Patagonian plateau in the south and the Gran Chaco in the north, over an area of some 10^6 km^2 (Nicolli et al., 1989; Smedley et al., 2001). The aquifers of the region are composed of deposits of loess, largely thought to have been deposited during the last glacial maximum, with some subsequent reworking. The sediments have an important component of intermixed volcanic ash of rhyolitic composition and contain abundant deposits of carbonate as layered and nodular calcrete. The study area covers around 110 $\times$ 70 km of northern La Pampa province, centred around the town of Eduardo Castex (35º55′S 64º18′W). A detailed account of the hydrogeochemistry of the region is given by Smedley et al. (2001).

Groundwaters are abstracted from a range of depths in the aquifers (6–140 m; Table 3). Groundwater chemistry is highly variable but salinity is often high. Many samples demonstrate the effects of evaporative concentration, particularly at the shallowest levels. Values for SO_4 and Cl have been recorded at up to 3170 mg l^{-1} and 4580 mg l^{-1} respectively (Table 3). The groundwaters are aerobic. As a result, concentrations of NO_3-N are often high, while NH_4-N, Fe and Mn are usually low (excepting a few samples where colloidal Fe is present). Many have high alkalinity (up to 1440 mg l^{-1}) as well as high pH values, often in excess of pH 8 (Smedley et al., 2001).

TABLE 3

Summary of groundwater quality in aquifers from La Pampa, Argentina

	Units	Min.	Max.	Median	*n*
Well depth	m	6	140	29.1	103
Temperature	ºC	16.1	29.1	19.8	108
pH		6.99	8.66	7.85	108
Eh	mV	131	492	325	102
DO	mg l^{-1}	0.8	9.9	6.1	106
SEC	μS cm^{-1}	773	17520	2610	108
Ca	mg l^{-1}	1.55	599	20.9	108
Mg	mg l^{-1}	2.01	521	21.5	108
Na	mg l^{-1}	120	3100	543	108
K	mg l^{-1}	3.3	70.6	11.8	108
Cl	mg l^{-1}	8.5	4580	192	108
SO_4	mg l^{-1}	6.8	3170	285	108
HCO_3	mg l^{-1}	195	1440	653	108
NO_3-N	mg l^{-1}	<0.2	144	9.06	108
NO_2-N	μg l^{-1}	<3	169	3	108
NH_4-N	mg l^{-1}	<0.01	0.14	<0.02	108
Si	mg l^{-1}	21.1	39.2	29.2	108
As(III)	μg l^{-1}	<3	105	4.2	108
As_T	μg l^{-1}	<4	5280	149	108
Fe_T	μg l^{-1}	<6	1160	55	108
Mn	μg l^{-1}	<1	79	2	108
V	mg l^{-1}	0.019	5.43	0.558	108
B	mg l^{-1}	0.46	13.8	2.97	108
P_T	mg l^{-1}	<0.2	0.7	<0.2	108
F	mg l^{-1}	0.034	29.2	3.84	108
Cu	μg l^{-1}	0.41	88.6	7.1	107
Se	μg l^{-1}	<2	8	40	34
Mo	μg l^{-1}	2.72	991	61.5	107
U	μg l^{-1}	6.16	248	30.6	107
$\delta^{13}C$	‰	−12.8	−4.0	−8.5	34

Abbreviations as for Table 1.

Concentrations of dissolved As_T range between <4 and 5280 μg l^{-1} (median 149 μg l^{-1}; mean 414 μg l^{-1}). Of the dissolved As present, measured As(III) concentrations form a very small proportion of the total, indicating that the dominant species is As(V). As(III)/As_T ratios lie predominantly in the range 0.02–0.06 (Fig. 8).

The concentrations of As_T are generally positively correlated with pH and alkalinity (Fig. 9; Smedley et al., 2001). Groundwater pH is considered to have been an important factor in the release of As as sorption of As to iron and other metal oxides is thought to be an important source of the As, and this sorption is also known to be strongly pH-dependent (Pierce and Moore, 1982; Dzombak and Morel, 1990). Desorption of As, principally as As(V), from iron oxides is more favourable in the groundwaters with the highest pH values.

Many other trace elements also reach high concentrations in the Pampean groundwaters. Concentrations of F reach up to 29 mg l^{-1}, V up to 5.4 mg l^{-1}, Mo up to 991 μg l^{-1}; U up to 248 μg l^{-1}, B up to 14 μg l^{-1} and Se up to 40 μg l^{-1} (Table 3). Many of these trace elements are also potentially detrimental to health and have assigned health-based WHO guideline values. As with arsenic, these also form anion or oxyanion species in solution and many display similar behaviour. Fig. 9 shows the positive correlations between As_T and F, V, B and Mo. These elements are also believed to be derived from iron oxides as well as aluminium oxides and clays (e.g., Dzombak and Morel, 1990; Goldberg, 1999) and from primary minerals such as apatite and micas.

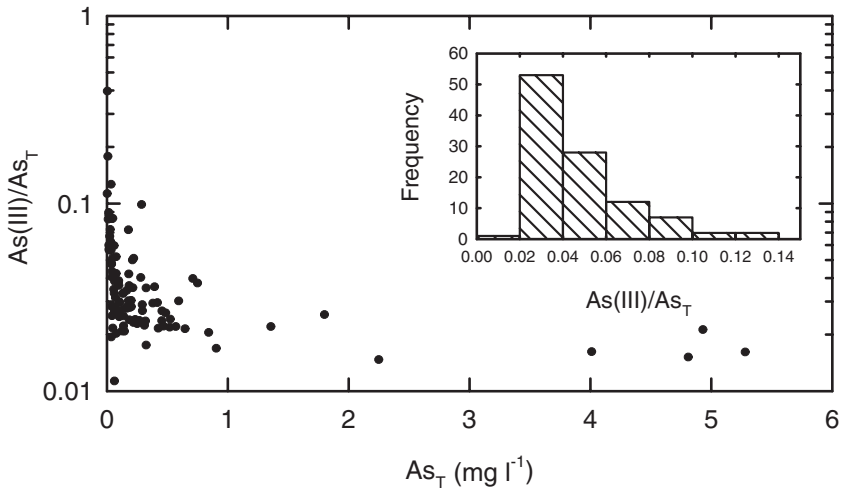

Fig. 8. Variation of As(III)/As$_T$ with As$_T$ in groundwaters from northern La Pampa, Argentina. Arsenic(III) is a very minor proportion of the total As present in solution; most is present as As(V).

DISCUSSION

The three case studies described show some important similarities and distinctions that serve to highlight the principal conditions under which As release into solution can take place. Clearly, mobilisation of As can be achieved over a wide range of redox conditions. Oxidising conditions with high pH values characterise the Argentine groundwaters, while strongly reducing conditions involving sulphate reduction characterise both the Bangladesh and Huhhot Basin groundwaters.

Documented evidence from other arsenic-affected aquifers worldwide demonstrates some notable similarities with the investigated study areas. Arsenic-bearing groundwaters from Taiwan, India, Bangladesh, Hungary, Romania, Vietnam and some parts of the USA for example typically also occur under strongly reducing conditions (e.g., Tseng et al., 1968; Welch et al., 1988, 2000; Varsányi et al., 1991; Berg et al., 2001). Indicators include lack of dissolved oxygen and nitrate, often low sulphate and high iron, manganese and ammonium concentrations. Much of the arsenic present in solution occurs as As(III). Some contain high concentrations of dissolved organic matter (e.g., Varsányi et al., 1991). Some aquifers are artesian (e.g., Tseng et al., 1968).

In contrast, groundwaters from arsenic-affected aquifers in Mexico, Argentina, Chile and parts of the USA (e.g., San Joaquin Valley, California; Fujii and Swain, 1995) are typically oxidising. In these, high pH (>8) is a common feature of the groundwaters. Oxidising groundwaters with arsenic problems typically contain high concentrations of dissolved oxygen, nitrate and sulphate (with low iron, manganese and ammonium concentrations). The arsenic is dominated by the oxidised form, As(V). Many have associated high concentrations of F, V, Se, Mo and some are brackish or saline (e.g., Del Razo et al., 1990; Fujii and Swain, 1995; Welch and Lico, 1998).

Many workers accept that the most important sources of As in aquifers are iron oxides and sulphide minerals (although oxides of manganese and aluminium and clay minerals have also been cited as possible additional sources; e.g., Brannon and Patrick, 1987; Goldberg and Glaubig, 1988). Release from iron oxides can be achieved by desorption under aerobic conditions and by desorption and dissolution under anaerobic conditions.

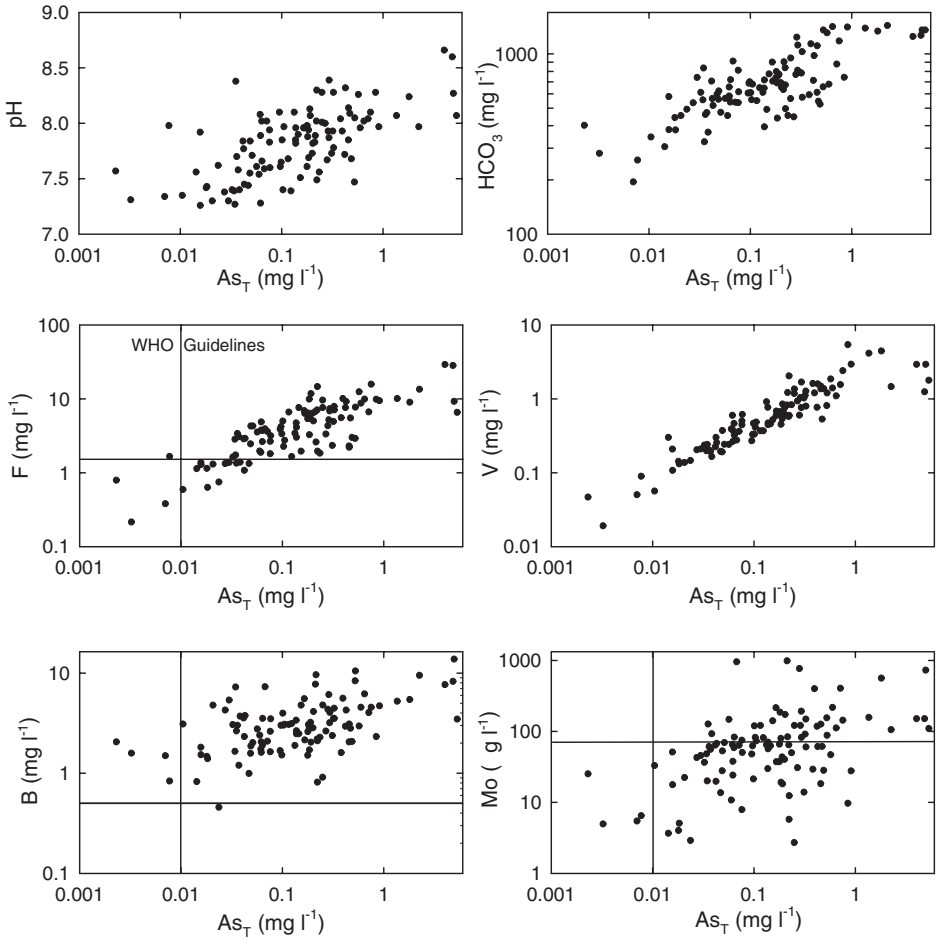

Fig. 9. Variation of pH, HCO_3, F, and V with As_T in the groundwaters from La Pampa. Dashed lines indicate concentrations of WHO guideline values where appropriate.

The mechanisms of release of As are still rather poorly understood in detail, but in aerobic conditions, desorption of arsenic (particularly As(V)) from iron oxides is known to be strongly pH-dependent (Pierce and Moore, 1982; Dzombak and Morel, 1990). Competition with other ions (e.g., PO_4 (Hiemstra and van Riemsdijk, 1999) and possibly HCO_3) can also reduce the sorption of As(V). Conditions of high groundwater pH are commonly, though not exclusively, found in arid and semiarid regions (e.g., Argentina, Chile, Mexico, southwestern USA) where groundwaters are dominantly of Na-HCO_3 type and where recharge is low and evaporation becomes important. Evaporation has been reported as an additional control on increasing As concentrations in groundwaters from some aquifers (Welch and Lico, 1998; Levy et al., 1999).

Under reducing conditions, As release is believed to be largely linked to reduction of As(V) on iron oxides to As(III), with resultant desorption, as well as reductive dissolution of the oxides themselves. The generation of strongly reducing conditions appears to have had a large impact on the As chemistry of the groundwaters of Bangladesh and

TABLE 4
Summary of groundwater and aquifer characteristics in the three arsenic-affected regions investigated

Region/Country	Aquifer	Groundwater chemistry	As source and mobilisation process	Groundwater flow
Bangladesh	Holocene alluvial/deltaic sand, silt, clay; much surface silt.	Strongly reducing: high Fe, Mn, NH_4, alkalinity. No DO, NO_3, low SO_4 – SO_4 reduction. As present as As(III) and As(V).	Fe oxides – desorption and dissolution.	Very slow; regional flow gradients low.
Huhhot Basin, Inner Mongolia	Holocene alluvial and lacustrine sands, silts, clays. Confined by impermeable sediments in centre of basin.	Strongly reducing. Moderate Fe, Mn, high NH_4, alkalinity. No dissolved oxygen, low SO_4, high humic-acid concentration. As present mainly as As(III).	Fe oxides – desorption and dissolution.	Slow in low-lying parts of the basin: 'closed basin'.
La Pampa, Argentina	Pleistocene and Holocene aeolian (loess) silts, unconfined.	Oxidising. Saline at shallow depths (evaporation). High pH, high alkalinity. As present as As(V). Also high F, V, B, Be, Se, U, Mo, P.	Fe (and likely Mn) oxides – desorption.	Slow; regional flow gradients low. Local discharge zones.

the Huhhot Basin. Processes such as ageing and restructuring of the iron oxides during sediment diagenesis may also be involved (Kinniburgh and Smedley, 2001). With the high solid/solution ratios found in aquifers (6–10 kg l^{-1}), even a small shift in the sorption isotherm can have a large impact on the concentration of As found in groundwater. Such shifts may occur due to a reduction of surface area of the oxide, or to a change in surface structure, perhaps related to the development of reducing conditions, as well as to changes in the oxidation state of the As. These processes may occur simultaneously and are poorly understood. It can therefore be difficult to isolate the dominant mechanism involved.

The other important sources of As in water are the sulphide minerals, of which pyrite is the most common, particularly in sediments. Oxidation of sulphide minerals is clearly the dominant control on mobilisation of As (and other trace elements) in mining areas. It has also been cited as the cause of As release in the aquifers of Bangladesh and West Bengal (e.g., Chowdhury et al., 1997), resulting from alleged irrigation 'overabstraction' and consequent dewatering of the aquifers. Oxidation of sulphide minerals requires the presence of an oxidising agent, such as oxygen, nitrate or ferric iron and one of the key products of the reaction is dissolved SO_4. The fact that high As concentrations in the Bangladesh (and Huhhot Basin) groundwaters have generally low SO_4 concentrations (typically 1 mg l^{-1} or less), indeed having negative correlations between As and SO_4, suggests that sulphide oxidation is not the dominant process involved in releasing As into the groundwaters of these aquifers. Instead, sulphate reduction involving *precipitation* of sulphide minerals is thought to have been important. Formation of authigenic iron monosulphides and pyrite is likely to remove many other elements from solution, including transition metals and to some extent As. This is the likely reason why concentrations of several other trace elements such as Cu are low. Most groundwaters from Bangladesh and reducing groundwaters from the Huhhot Basin have Cu concentrations <1 $\mu g\ l^{-1}$ (Tables 1 and 2).

A number of key features are common to each of the studied areas reported. The characteristics of each are summarised in Table 4. Concentrations of total As found in

the sediments in each study area are not unusually high: the range for total As observed in Bangladesh sediments is 0.4–15 mg kg^{-1} (Kinniburgh and Smedley, 2001), that for sediments from the Huhhot Basin is 3–29 mg kg^{-1} (Smedley et al., 2000) and sediments from La Pampa have a range of 3–18 mg kg^{-1} (Smedley et al., 2001). In each study area, iron oxides are believed to be important sources of the As in the groundwaters. The role of manganese oxides in the aquifers of La Pampa is not clear, although they are also believed to play a role as they are relatively abundant in the sediments.

All the aquifers are relatively young (Quaternary, many Holocene) and groundwater flow is in each known to be relatively sluggish as a result of various combinations of factors such as arid conditions, low recharge rates, low hydraulic gradients and poorly permeable sediments. Lack of aquifer flushing over the short history (of the order of a few thousand years) of the aquifers is therefore thought to have been important to the generation of groundwater As problems, as there will have been less possibility of removal of As and other trace elements from the aquifer. Older aquifers are thought to be less at risk from groundwater As contamination, even under reducing conditions or oxidising, high-pH conditions.

CONCLUSIONS

Mobilisation of As in waters occurs in a range of geochemical environments. These can be mainly divided into (1) aquifers under strongly reducing conditions, (2) aquifers under oxidising, high-pH (>8) conditions, (3) areas of sulphide mineralisation (especially mining areas), and (4) geothermal areas. The most extensive and potentially most detrimental to human health are the major aquifers which are often relied upon heavily for potable supply. In the As-affected aquifers investigated, other key factors appear to be the young ages of the aquifer sediments, i.e., Quaternary, and slow groundwater flow rates such that aquifer flushing will have been less advanced. Arsenic problems in major aquifers are therefore prevalent where geochemical conditions are favourable for arsenic mobilisation in water but where hydrogeological conditions are unfavourable for its removal from the aquifer system.

In regions with oxidising groundwaters, potentially useful indicators of arsenic problems include high pH values (>8) and high alkalinities (>500 mg l^{-1}). Ancillary information such as prevalence of dental fluorosis in local populations is also useful as it suggests occurrence of high fluoride concentrations in the drinking water which, as described, is often associated with As. In regions with reducing groundwaters, useful indicators are the occurrence of high Fe, Mn and NH_4 concentrations and often high P concentrations. As with the oxidising groundwaters, high alkalinity is a common characteristic of As-affected groundwaters.

The characteristics of geologically vulnerable aquifers outlined lead to the question of whether other areas in the world may suffer arsenic problems in groundwater, but have not yet been identified because of lack of testing for arsenic and/or lack of observed As-related health problems. The aquifers most 'at risk' are taken to be those in Quaternary sediments, particularly in large low-lying alluvial and deltaic basins, as well as large loess plains. In each case, slow groundwater flow and poor mixing, as reflected by highly variable groundwater quality, are also indicators. Potential examples include the large deltas (e.g., Irrawaddy delta of Myanmar, Indus valley of Pakistan, Mekong valley of Vietnam and Cambodia, Nile delta of Egypt). In these areas, the fresh groundwaters are likely to be of greater concern because those of higher salinity close to the coast are not likely to be used for potable supply. Alluvial and loess plains such as those in large parts of northern China as well as Paraguay and Uruguay are also potentially vulnerable. For such areas, the rapid surveying of groundwater resources for As to assess risks and scales of contamination should become an urgent priority.

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Arsenic Exposure and Health Effects IV
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Characterization of Arsenic-bearing Sediments in the Gangetic Delta of West Bengal, India

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ABSTRACT

In the Ganges–Meghna–Brahmaputra (GMB) delta of West Bengal, India, and neighboring Bangladesh ingestion of arsenic-contaminated groundwater has caused serious and widespread human health effect. In West Bengal about 55% and 34% of 90,000 hand tubewells we had so far analyzed from affected districts by FI–HG–AAS showed arsenic values above 10 μg/l and 50 μg/l, respectively, while in Bangladesh the values were 73% and 59%, respectively, out of a total of 27,000 samples we had analyzed so far jointly with the Dhaka Community Hospital. Altogether over 100 million people are potentially at risk in these two countries. The source of arsenic is geologic. Examination of the sediments ($n = 2235$) from 112 boreholes showed that 85 samples contained arsenic 10–196 mg/kg and opaque particles separated from the parent sediments contained arsenic up to 2778 mg/kg. Mineralogical studies from several laboratories clearly show that both pyrite and iron oxide contain very high concentrations of arsenic. If oxidation of pyrite is the source of arsenic in groundwater then there are two questions to be answered: (a) whether arsenic-rich pyrite is sufficient to account for the mass of arsenic mobilized in groundwater; and (b) whether increased pyrite oxidation has led to increased arsenic and sulfate concentrations. Ore microscopic examination using reflected light indicates abundant highly reflective opaque particles, suggesting the presence of sulfide minerals. Electron microprobe analysis of the pyrite particles revealed arsenic contents ranging from 0.07 to 1.36 wt%. Gypsum may be a product of oxidation of sulfur in pyrite to sulfate followed by dissolution to calcite common to borehole samples studied. We have also identified framboidal and other secondary pyrite; this might partially explain the low sulfate concentrations in groundwater. Interviews of thousands of old people in affected area lead us to conclude that arsenical skin lesions were not present in those areas before the early eighties. Also, we have found that initially low-arsenic groundwater from some tubewells now contains unsafe arsenic concentrations. We believe that a single mechanism is not responsible for the release of arsenic from groundwater aquifer sediments in this region.

Keywords: groundwater arsenic contamination in GMB-delta; regional setting; borehole sediment analysis using XRD, EPMA, SEM, LAMMA; arsenic-rich pyrite; probable mechanism of arsenic leaching from source to aquifer

INTRODUCTION

Out of 20 countries in different parts of the world where groundwater with arsenic contamination and suffering of people have been so far reported, the magnitude is considered highest in Bangladesh followed by West Bengal, India (Chatterjee et al., 1995; Das et al., 1995; Biswas et al., 1998; Mandal et al., 1998a,b; Chowdhury et al., 2000; Smith et al., 2000).

From our about 90,000 hand tubewells water analyses from West Bengal, India, and about 27,000 from Bangladesh we found that 9 districts of West Bengal, India, out of its total of 18 and 47 districts of Bangladesh out of a total of 64 have arsenic levels in groundwater above the World Health Organization maximum permissible limit of 50 μg/l. The area and population of 47 districts in Bangladesh and 9 districts of West Bengal are 112,407 km^2, 93.5 million and 38,865 km^2, 42.7 million, respectively. However, this does not mean that 136.2 million people in Bangladesh and West Bengal are drinking contaminated water above 50 μg/l but no doubt they are at risk. DPHE Bangladesh and the British Geological Survey (British Geological Survey, 1999) out of its 2000 hand tubewells water samples from eastern, southern and western parts of Bangladesh reported that approximately 21 million people in Bangladesh were drinking arsenic-contaminated water above 50 μg/l. The magnitude of arsenic contamination in Bangladesh surfaced only after the international conference on arsenic in Dhaka, Bangladesh, in February 8–12, 1998. A report (Pearce, 1998) described the magnitude of the arsenic contamination in Bangladesh: the World Bank's local chief stated that tens of millions of people are at risk from health effects and that 43,000 of the 68,000 villages are presently at risk or could be at risk in the future. In the same report (Pearce, 1998), the World Health Organization (WHO) predicted that, within a few years, 1 in 10 adult deaths across much of southern Bangladesh could be from cancers triggered by arsenic.

Our earlier report (Mandal et al., 1996) stating that a single Rural Water Supply Scheme (RWSS) (Mothabari, Kaliachalk-II, Malda district, West Bengal, India) is alone withdrawing 147 kg of arsenic from groundwater in a year, and 6.4 tons of arsenic is coming out in a year from 3000 shallow big-diameter tubewells in use for agricultural irrigation in Deganga Block, 24-Parganas (North), West Bengal (Mandal et al., 1998b), indicate that the source of arsenic is not anthropogenic; most probably the source is geologic. Although the source of the aqueous arsenic is believed to be aquifer sediments, the chemistry and mineralogy of the sediments of the Ganges–Brahmaputra–Meghna (GBM) delta are not well understood. The potential impact of water management on the aqueous arsenic concentrations requires an understanding of the sediments. As a result, it is felt necessary to study the borehole sediment samples of the arsenic-affected areas of West Bengal to find out the geochemical environment and the chemistry involved in leaching out of arsenic from the mineral phase and their mode of occurrence and origin.

During 1996 we reported (Das et al., 1996) that the source of arsenic in Gangetic West Bengal is geologic and our borehole sediment analysis showed the existence of arsenic-rich pyrite (FeS_2). A probable explanation we indicated for arsenic contamination of groundwater is the breakdown of arsenic-rich pyrite, which occurred due to a change of the underground geochemical environment by the withdrawal of unlimited amounts of groundwater. We also stated that due to heavy groundwater withdrawal the underground aquifer is aerated and oxygen causes degradation of pyrite, the arsenic-rich source. During 1998 Bhattacharya et al. (Bhattacharya et al., 1998) predicted that the cause of arsenic contamination of groundwater is due to reduction of arsenic-rich iron oxy-hydroxide in anoxic groundwater. Later Nickson et al. (Nickson et al., 1998, 2000) supported the idea of Bhattacharya et al. and also reported that arsenic concentration increases with depth (Nickson et al., 1998). This was against our findings of 7800 water samples from Bangladesh and 55,000 water samples from West Bengal of known depth of arsenic in groundwater (Roy Chowdhury et al., 1999). During May–August 2000, a large number of

researchers further discussed the sources of arsenic and mechanisms of leaching focusing on pyrite oxidation and iron oxy-hydroxide reduction (Harvard University, USA, 2000). Recently, high arsenic concentrations in groundwater have been reported (Schreiber et al., 2000) from Wisconsin, USA. Arsenic occurs in pyrite and marcasite as well as in iron oxy-hydroxides. It has been reported that sulfide oxidation is the dominant process controlling arsenic release to groundwater. It has also been reported that although arsenic contamination is caused by oxidation of naturally occurring sulfides, it is influenced by water level fluctuations caused by municipal well pumping or climate changes.

The present paper will report the characterization of arsenic-bearing sediments in the Gangetic delta of West Bengal. We have analyzed 2235 borehole sediment samples from 112 boreholes and 1 core boring from 6 out of 9 arsenic-affected districts of West Bengal. To characterize the arsenic-rich sediments we have used petrology, mineralogy, X-ray diffraction (XRD), scanning electron microscopy (SEM), electron probe microanalysis (EPMA), and laser microprobe mass analysis (LAMMA). A probable mechanism of arsenic release has also been discussed in this report.

REGIONAL GEOLOGY

The area constitutes the southern part of the Bengal Basin, a pericratonic basin in north-eastern India. The basin which comprises the Ganges–Brahmaputra delta in its southern part has had a long evolutionary history since its formation and is the result of the break-up of Gondwanaland along its rifted passive margin of the Indian Plate and its subsequent northerly movement during the Early Cretaceous (126 m.y. ago). The initial collision of the Indian and Eurasian plates began in the Early Eocene (41–40 m.y. ago) and resulted in the initial uplift of the Himalayas. As a result of this convergence, the Ganges delta and the proto-Brahmaputra delta sediments were juxtaposed and gradually merged. Relatively recent folding and uplift (Quaternary epoch) of the Brahmaputra sediments close to the intraplate boundary redirected the course of the Brahmaputra to its present configuration (Morgan and McIntyre, 1959; Lindsay et al., 1991). The Ganga–Brahmaputra delta complex, which thickens towards the south, has three broad stratigraphic sequences viz.: (1) the proto-Ganges delta formed immediately after the break-up of Gondwanaland (126 m.y. ago): (2) the Transitional delta (49.5–10.5 m.y. ago); and (3) the Modern delta (10.5 m.y. ago–present) with a successive sequence of sands, sandy clay, silt and clay which were deposited under a major eustatic sea level low at about 10.5 m.y. ago. The modern delta has been formed primarily of alluvial sediments transported by the rivers, e.g., the Mayurakshi, the Ajoy, the Damodar, etc., originating from the Chotonagpur Uplands in the west and subsequently by the rivers flowing from the Himalayan foredeep basin, e.g., the Ganges, the Padma, the Bhagirathi, the Brahmaputra, etc., flowing from the north when a gap, the Garo–Rajmahal gap, was created due to tectonic movements (Auden, 1949). In West Bengal arsenic-contaminated groundwater occurs mostly in the eastern side of the River Bhagirathi.

GEOMORPHOLOGY

The modern delta can be divided into two macroform processes regions: (1) the upper delta plain of meander belts of the Padma–Bhagirathi rivers in the north; and (2) the lower delta plain with several tidal creeks in the south. The upper delta plain is characterized by a series of meander scars of various wavelengths and amplitudes, abandoned channels, Ox-bow lakes, formed under varying hydrodynamic conditions in a fluvial regime. Abandoned meander scrolls are the most common form and could be related to flood-plain formation in the upper delta plain with a very gentle southerly slope. Other landforms are levees, backswamps in between inter-distributary levees, etc. The sediments of this plain

consist of several fining-upward sequences (clay–silt–sand). Such cyclothems in the form of festoons are manifestations of the shifting of the meander belts with channel lag bars, levees and backswamp sediments representing the coarse- to medium-grained sand, fine sand and silty clay, respectively. The lower deltaic plain constitutes tidal mudflats, distributary levees and interdistributary marsh complexes formed under a fluvial–estuarine–marine environment under the influence of fluctuating sea-level conditions in geologically recent times (Pleistocene–Holocene). The lower deltaic plain is characterized by the presence of an extensive clay capping of varying thickness (15–76 m) which is underlain by silt, sand and gravel in many places (Deshmukh and Goswami, 1973). Areas affected by arsenic contamination in groundwater are all located in the upper delta plain and mostly in the abandoned meander belts.

GEOHYDROLOGY

The shallow aquifer (12–15 m below ground level, bgl) in the upper delta plain is mostly under unconfined conditions except near its southern fringe where it occurs under semi-confined to confined conditions. There are two more aquifers with depths ranging from 35 to 46 m and 70 to 150 m in the districts of Malda, Murshidabad, Nadia and Bardhaman and North 24-Parganas. In the South 24-Parganas these aquifers occur at greater depths. There is generally a southeasterly gradient of the water surface sub-parallel to the general slope of the area. All the aquifers are interconnected due to spatial variations in grain size. In general, the upper aquifer material comprises sub-rounded to sub-angular fine- to medium-grained sand with occasional clay lenses. Its heavy mineral assemblage (opaque, altered biotite, garnet, tourmaline, kyanite, zircon) indicates a mixed metamorphic-cum-igneous provenance. The intermediate aquifer, constituted of sub-angular to sub-rounded medium sand, sandy clay and clay with fine sands, and its heavy mineral assemblage (biotite, garnet, kyanite, opaques), indicates a dominantly metamorphic origin. While the lower aquifer is constituted of sub-rounded to rounded fine to coarse sand with occasional clay bodies, and its heavy mineral assemblage (opaques, altered biotite tourmaline, rutile, etc.) indicates an igneous provenance (PHE report, Government of West Bengal, 1991). The intermediate aquifer usually shows arsenic contamination. At greater depth, aquifer arsenic may not be present at the beginning but may become contaminated in the course of time. This is what we have observed during our study over the last 12 years.

BOREHOLE SEDIMENT SAMPLE

The borehole sediment samples were collected during drilling of wells from the districts Malda, Murshidabad, Nadia, North 24-Parganas, South 24-Parganas and Calcutta (six out of the nine arsenic-affected districts). Most of the samples were collected during drilling through Public Health Engineering Department (PHED), Govt. of West Bengal, Central Drilling Division, PHED, Govt. of West Bengal and Central Ground Water Board (CGWB), Govt. of India. A few drillings were financed by us including a core-boring. Samples were collected at every 3.03 m or 6.06 m interval. The samples were collected up to a depth of 250 m. Three techniques were used for drilling. They were: (1) hydraulic rotatory drilling; (2) manual drilling; and (3) core drilling. Details about the drilling are described elsewhere (Chatterjee et al., 1992; Roy Chowdhury, 1999).

SAMPLE PROCESSING

The borehole sediment samples were dried in open air under diffuse sunlight followed by drying in an oven at 50ºC. The sediment samples were divided into three parts: (a) parent

sample (P); (b) homogenized sample (H); the parent sediment sample was powdered using agate mortar and pestle; and (c) selected particle (S) (these particles are opaque, soft or hard, reddish or brown in nature and were picked from the parent sample with naked eye or pocket lens).

METHODOLOGY

A large number of borehole sediment samples were analyzed for arsenic and other heavy metals using atomic absorption spectrometer (Perkin Elmer Model 3100 and Varian AAS Model Spectra AA-20), both GFAAS and FI–HG–AAS, X-ray fluorescence, and ICP–AES. Sediment samples were examined and analyzed by binocular microscope, petrological microscope, X-ray powder diffractometer, electron probe micro-analyzer (EPMA), laser microprobe mass analyzer (LAMMA), and scanning electron microscope (SEM). Details of the procedures of sample preparation and instrumentation have been described in our earlier publications (Chatterjee et al., 1992, 1995; Das et al., 1996; Roy Chowdhury, 1999).

RESULTS AND DISCUSSION

The locations of the police stations/blocks in the arsenic-affected districts of West Bengal from where borehole sediment samples were collected is presented in Fig. 1. The overall study reports on the number of boreholes from each district, number of samples analyzed, etc., are shown in Table 1. The sediment samples were collected at a maximum depth of up to 250 m from all the six affected districts except in Malda where we could not drill below about 100 m as we encountered hard rock. Arsenic concentration in the parent sediment (P) or homogenized (H) form of the parent sample may differ, but the selected opaque particles from the parent sample always contain elevated levels of arsenic. Arsenic concentration is not depth dependent in sediment samples. Higher concentrations may be found at any depth. Fig. 2 shows arsenic concentrations in parent, homogenized and selected particles at different depths from some borehole samples in the Malda and Murshidabad districts.

After analyzing aliquots from 2235 borehole sediment samples without homogenizing we found 86 samples containing arsenic in the range of 10–373 mg/kg and this number decreased to 48 after being homogenized. The homogenized samples show arsenic concentrations of 10–196 mg/kg. Selected particles from parent samples show arsenic concentrations as high as 2780 mg/kg. Fig. 3 represents the abundance of different sediment fractions in borehole sediment samples ($n = 1070$) in surveyed districts. The average concentration of arsenic varies in the order coarse grain > fine to medium sand > clay–clay mix with fine sand, but the abundance of arsenic is highest in clay–clay mix with fine sand.

Table 2 represents the location, depth and concentration of arsenic and heavy metals Fe, Cu, Pb, Ni, Cr, Mn and Zn in ten sediment samples having arsenic concentrations higher than 10 mg/kg in homogenized samples. Standard Reference Materials were analyzed for the validity of the result (Table 3). Regression analyses have been carried out between the arsenic and other heavy metals, e.g., Fe, Cu, Pb, Ni, Cr, Mn and Zn in 43 homogenized samples having arsenic concentrations above 10 mg/kg. The linear regressions show good correlations between arsenic and iron ($r^2 = 0.95$, $P < 0.001$) and arsenic and lead ($r^2 = 0.92$, $P < 0.001$). Good correlations were seen in some individual boreholes, e.g., in the borehole of Mahisbathan village, Karimpur, Nadia district. Also, good correlations were observed between As and Cu ($r^2 = 0.808$, $P < 0.001$), As and Ni ($r^2 = 0.818$, $P = 0.001$), and As and Cr ($r^2 = 0.56$, $P = 0.0086$). The identical values of the As/Fe ratio of the borehole sediment samples of eight consecutive layers in borehole samples of Mahisbathan village indicate that both iron and arsenic are coming out from a definite source rich in

TABLE 1

Study report of borehole sediment samples in arsenic-affected districts of West Bengal, India

District	24-Parganas (South)	24-Parganas (North)	Nadia	Murshidabad	Malda	Calcutta	Total
No. of blocks/p.s	2	4	5	5	1	1	18
No. of boreholes examined	5	14	75	13	4	1	112
No. of samples analyzed	108	620	1000	314	130	63	2235
No. of boreholes found arsenic conc. > 10 mg/kg	2	8	20	9	2	Nil	41
No. of samples found arsenic conc. > 10 mg/kg (P)	2	24	41	15	4	Nil	86
No. of samples found arsenic conc. > 10 mg/kg (H)	2	10	25	7	4	Nil	48
Maximum conc. of arsenic found (mg/kg)	16.88 (P)	60.50 (P)	373.80 (P)	77.60 (P)	122.47 (P)	4.44	
	12.81 (H)	69.47 (H)	196.75 (H)	31.19 (H)	208.80 (H)		
		413.17 (S)	2777.78 (S)	2116.53 (S)			
Depth at which maximum conc. found (m)	41.81–97.27	208.02–211.02	136.36–139.39	136.36–151.51	78.78–81.81	44.24–47.27	

Note: P = parent sample; H = homogenized sample; S = selected particle from parent sample.

TABLE 2

Concentration of arsenic and other heavy metals in borehole sediment samples from Haspukuria village, Block Tehatta, district Nadia of West Bengal

Sample No.	Depth (m)	Characteristics of borehole sediment	As (mg/kg) before homogenizing	Concentration of heavy metals, being homogenized (mg/kg)							
				As	Fe	Cu	Pb	Ni	Cr	Mn	Zn
BH56	65.45–67.27	Coarse sand	20.72	64.39	48299.84	15.46	73.42	30.91	19.32	2279.75	77.28
BH57	67.27–69.09	Coarse sand	15.72	113.53	27810.30	15.22	42.15	24.59	12.88	948.48	46.84
M-38	112.12–115.15	Fine to very coarse sand with rock fragment	27.04	69.72	18474.62	2.79	48.80	12.55	–	1993.87	27.89
M-40	118.18–121.21	Medium to very coarse sand with rock fragment	22.71	41.08	31296.95	1.25	11.27	11.27	–	2879.32	25.04
M-41	121.21–124.24	Very coarse sand with rock fragment	6.50	156.47	38878.42	3.53	45.95	14.14	–	3534.40	23.56
M-42	124.24–127.27	Fine to very coarse sand with rock fragment	59.45	71.61	19259.64	8.69	50.57	8.69	2.37	1430.15	23.70
M-45	133.33–136.36	Fine to medium sand with small black particles	74.51	52.84	7479.19	1.30	70.24	9.11	–	143.08	26.01
M-46	136.36–139.39	Very coarse sand with rock fragment	373.80	196.75	31971.41	11.80	212.49	20.99	9.18	1036.20	26.23
M-47	139.39–142.42	Medium to very coarse sand with rock fragment	103.28	181.41	30430.71	1.87	44.00	14.98	2.81	1779.03	9.36
M-48	142.42–145.45	Clay mixed with very coarse sand	24.17	141.28	34536.17	10.05	123.91	36.84	18.42	1088.41	33.49

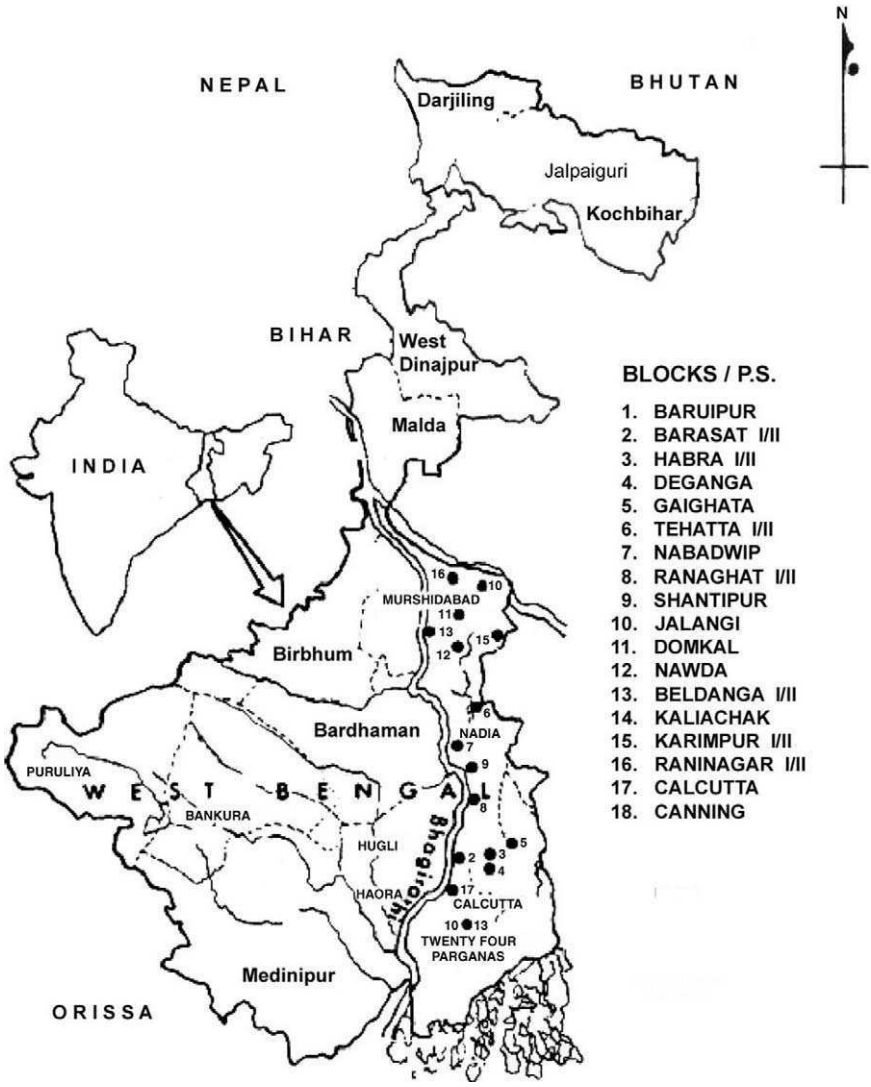


Fig. 1. The locations of the police stations/blocks in the arsenic-affected districts of West Bengal from where borehole sediment samples were collected.

arsenic and iron. The statistical presentation is given in Table 4. Similar findings were observed in many other borehole sediment samples.

Core-boring at Kolsur Village, Deganga Block, 24-Parganas (North) District

During our analysis of the water from Kolsur village we found high concentrations of arsenic in many samples (maximum concentrations up to 1000 μg/l) and 70% of the samples contain arsenic above 50 μg/l. A core-boring was done in the area where a high arsenic content in the sediment was expected. The core boring was done up to 52.45 m with samples collected from each 0.32 m interval. Eleven layers had been found

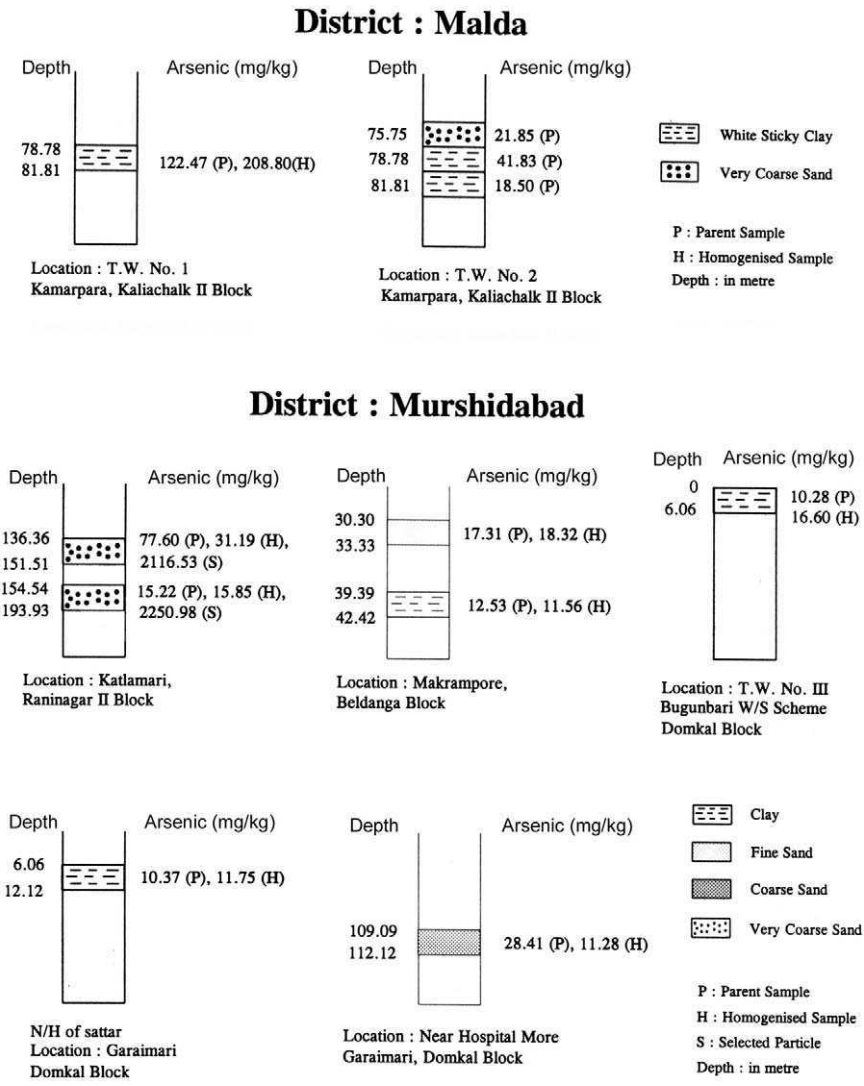


Fig. 2. Arsenic concentration in parent homogenized and selected particles at different depths from some borehole samples in Malda and Murshidabad districts.

with arsenic concentrations between 8 and 12 mg/kg at depths between 1 and 39 m. Regression analysis was carried between arsenic and iron concentrations ($n = 60$) and shows a good correlation between arsenic and iron ($r^2 = 0.89$, $P < 0.010$). During drilling the water samples were collected at 15.15 m and 30.30 m below ground level for the measuring of arsenic and iron in groundwater. The concentrations of arsenic and iron in the water samples from 15.15 m and 30.30 m were 215 μg/l and 3120 μg/l and 166 μg/l and 2110 μg/l, respectively. Although we expected high arsenic-rich sediment in the core-boring area due to a high abundance of arsenic in groundwater found during our earlier survey in the area, the results show that the sediments are not rich in arsenic.

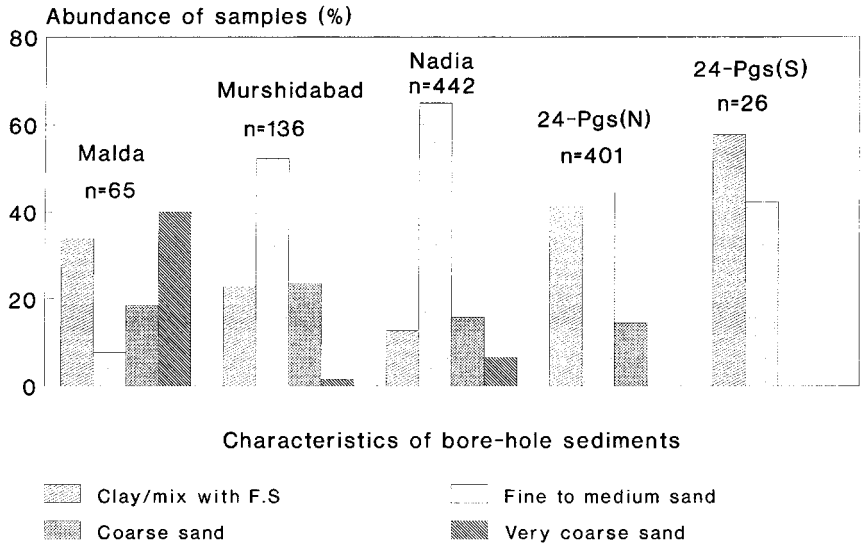


Fig. 3. Abundance of different sediment fractions in borehole sediment samples collected from districts of West Bengal.

Similar observations were encountered in other cases also. Thus it may be assumed that the arsenic-rich water may be flowing from a distant source and that it is not necessarily the higher concentration of arsenic in the aquifer that will cause a higher amount of arsenic in borehole sediment.

Arsenic-rich Selected Particles (S) from Borehole Sediments

During our borehole sediment analysis we had observed that usually in arsenic-rich parent sediment samples some blackish, reddish or brown-colored particles, opaque, and soft or hard in nature are present and some of them have a metallic hue. Most of these particles are found to be arsenic-rich. From arsenic-rich parent sediment samples we picked these particles by hand or simple magnifying glass. These particles are named 'selected particles (S)'. Table 5 represents the study report of selected particles from 11 boreholes along with concentrations of arsenic and other heavy metals. The arsenic-rich selected grains are also iron-rich. The other heavy metals, e.g., Fe, Pb, Ni, Mn, Cu and Zn content, are also high for selected grains compared to the arsenic-rich parent borehole sediment samples (being homogenized). The regression analyses carried out between arsenic and other heavy metals, e.g., Fe, Cu, Pb, Ni, Cr, Mn, showed good correlations between arsenic and iron ($r^2 = 0.90$, $P < 0.001$) and arsenic and lead ($r^2 = 0.81$, $P < 0.001$). The arsenic/iron ratio of B, D, 62A and 12X samples are 0.0082, 0.0100, 0.007 and 0.0086, respectively. The identical values (mean = 0.0086, median = 0.0084, std. dev. = 0.00098) indicate that both arsenic and iron are coming from a source rich in arsenic and iron.

Petrological Study of Selected Grains

Systematic sedimentological study of some high arsenic-containing sediment samples have been carried out using a binocular microscope and under a petrological as well as ore microscope. Some selected particles (S) show characteristic features when observed under a binocular microscope with reflected light. A golden–yellow color is sporadically distributed throughout the body of the rock fragment, which appears to be 'fools gold' or pyrite

TABLE 3

Concentration of heavy metal in standard reference materials (μg/g)

Heavy metals	Certified value	Observed value
Sample NIES-2: pond sediment		
Cu	210 ± 12	218 ± 8
Pb	105 ± 6	100 ± 1.2
Ni	40 ± 3	37 ± 6
Cr	75 ± 5	81 ± 3
Mn	770	710 ± 5
Zn	343 ± 17	345 ± 12
Sample CRM 038: fly ash from pulverized coal		
Cu	176 ± 9	170 ± 13
Pb	262 ± 11	250 ± 2.5
Ni	194	190 ± 6
Cr	178	182 ± 5
Mn	479 ± 16	490 ± 8
Zn	581 ± 29	600 ± 30.6
Fe	33800 ± 700	34140 ± 695
Sample NIST-1645: river sediment		
Cu	109 ± 19	116 ± 13
Pb	714 ± 28	698 ± 32
Ni	45.8 ± 2.9	46.8 ± 3.6
Cr	2.96 ± 0.28%	3.10 ± 0.2%
Mn	785 ± 97	799 ± 69
Zn	1720 ± 169	1705 ± 201
Fe	11.3 ± 1.2%	10.9 ± 1.5%
Sample BCR-146: sewage sediment		
Cu	934 ± 24	898 ± 40
Pb	1270 ± 28	1206 ± 50
Ni	280 ± 28	290 ± 10
Cr	784	802 ± 22.2
Mn	588 ± 24	564 ± 30
Zn	4059 ± 90	4016 ± 77

(Fig. 4). Clusterings of some quartz particles cemented by the ferruginous matrix (Fig. 5) have been found to contain high arsenic concentrations.

A study of bulk core-boring sediment samples from a high arsenic-rich groundwater area under a petrological microscope revealed that a considerable amount of opaque particles are present in the sediment (Figs. 6 and 7).

Polished Section Study

A polished section study under an ore microscope of some arsenic-rich particles from a sludge sample which appear under the binocular microscope as 'fools gold' (pyrite) reveals that huge amounts of pyrite grains (Fig. 8) are embedded in argillo-calcareous rock. Rhombic-shaped grains of calcite have also been found.

MINERALOGY AND X-RAY DIFFRACTION STUDY

Mineralogy and X-ray diffraction studies (XRD) of some selected particles were carried out. Some of the results are presented in Table 6. These observations revealed the presence of pyrite grains, iron oxide, quartz, white chalky grains (calcite). An elaborate report of the sediment S-70 from Sethpur village, Barasat-1 police station, North 24-Parganas district is given below.

TABLE 4

Statistical presentation of As/Fe ratio in borehole sediments of Mahisbathan village, Karimpur-II Block, Nadia

Sample No.	Depth (m)	As (mg/kg)	Fe (mg/kg)	As/Fe	Statistical presentation of As/Fe in borehole sediment) ($n = 8$)				
					Mean	Minimum	Maximum	Median	Std. dev.
M-38	112.12–115.15	69.72	18474.62	0.0038					
M-41	121.21–124.24	156.47	38878.42	0.0040					
M-42	124.24–127.27	71.61	19259.64	0.0037					
M-43	127.27–130.30	96.76	18061.93	0.0054	0.0047	0.0037	0.0061	0.0043	0.00097
M-44	130.30–133.33	27.57	5967.21	0.0046					
M-46	136.36–139.39	196.75	31971.41	0.0061					
M-47	139.39–142.42	181.41	30430.71	0.0059					
M-48	142.42–145.45	141.28	34536.17	0.0041					

Fig. 4. Argillo-calcareous rock fragment containing huge amounts of pyrite particles.

Sample S-70 has been examined mineralogically to identify the phases, which build up the sediment. The whole sample has been separated into different fractions under the microscope and with a permanent magnet by distinct morphologic and magnetic properties.

TABLE 5

Study report of arsenic-rich selected particles from borehole sediments

Sample No.	Source	Location	Depth (m)	Characteristics of parent sample	Concentration of parent sample (mg/kg)		
					As before homog-enizing	As being homog-enized	Fe being homog-enized
A		Block Kaliganj, Nadia	60.00–70.00	Medium to coarse sand with yellowish and blackish particles	<10.00	NA	NA
B	BH10	Katlamari, Block Raninagar-II, Murshidabad	154.54–193.93	Very coarse sand with rock fragments	15.22	15.85	13567.93
D	BH26	Katlamari, Block Raninagar-II, Murshidabad	136.36–151.51	Very coarse sand with rock fragments	77.60	31.19	17717.50
E		Block Santipur, Nadia	80.00–90.00	Medium sand, white with biotite and mica	32.62	<10.00	NA
HK1		By the side of the house of Chitta Ranjan Roy, Kankpul, Block Habra-I, 24-Pgs (North)	172.72–175.75	Medium sand	<10.00	NA	NA
T		Block Nabadwip, Nadia	90–100	Very coarse sand with rock fragments	<10.00	NA	NA
62A	BH62	Gadigacha, Block Nabadwip, Nadia	218.18–221.21	Very coarse sand with rock fragments	85.72	22.07	12552.10
12X	M46	Mahisbathan, Block Karimpur-II, Nadia	136.36–139.39	Very coarse sand with rock fragments	373.80	196.75	31971.41
12Y	M46	Mahisbathan, Block Karimpur-II, Nadia	136.36–139.39	Very coarse sand with rock fragments	373.80	196.75	31971.41
12YH	M46	Mahisbathan, Block Karimpur-II, Nadia	136.36–139.39	Very coarse sand with rock fragments	373.80	196.75	31971.41
S-70		Sethpukur, Barasat, Block Barasat-I, 24-Pgs (North)	208.02–211.02	Coarse to very coarse sand	60.50	69.47	NA

Note: BDL = below detection limit; NA = not analyzed.

These fractions can be characterized as follows.

Fraction A: strong magnetic, opaque, dark brown to black-colored elongated aggregates, often with semi-spherical surface, constitute a very small fraction of the total sediment.

Fraction B: opaque, yellow crystals often agglomerated into rods, also constitute a very small fraction of the total.

Characteristics of selected particles	Concentration of heavy metals in selected particles (mg/kg)							
	As	Fe	Cu	Pb	Ni	Cr	Mn	Zn
Reddish grain	130.98	155075.19	56.39	225.56	93.98	131.58	1503.76	375.94
Black, grey grain	2250.98	272606.38	6.65	212.77	186.17	BDL	332.45	132.98
Black, grey grain	2116.53	206984.56	64.10	641.02	256.41	BDL	BDL	641.03
Blackish particles	195.86	130714.98	29.76	297.62	59.52	BDL	BDL	297.62
Brownish and blackish small particles	115.67	131578.95	54.82	208.30	76.75	76.75	9978.07	219.30
Brownish and blackish particles	148.61	172505.31	47.77	159.24	74.31	74.31	636.94	159.24
Blackish particles with metallic shining	2292.80	298913.04	14.88	168.65	128.97	BDL	BDL	99.21
Blackish particles with metallic shining	2777.78	322745.90	24.14	403.00	81.96	BDL	BDL	241.0
Blackish particles, no metallic shining	2169.76	113695.43	15.59	1299.38	187.11	88.36	103.95	51.98
Blackish particles, no metallic shining	1187.39	57339.45	22.94	412.84	137.61	103.21	229.36	114.68
Blackish with metallic shining	413.17	353700.00	38.90	–	28.64	196.12	16800.00	67.77

Fraction C: the residual of the separation consists of light, rounded grains, which are about 95–98% of the total sediment.

These different fractions have been powdered and examined with a Siemens D 5000 powder diffractometer using Cu-target and an excitation condition of 45 kV and 39 mA. The scattered X-rays have been split by graphite secondary-monochromator in front of the counter. The phases have been identified by the software supplied with the instrument,

Fig. 5. Clustering of quartz and mica grains coated by a ferruginous matrix.

called DIFFRACT version 3.0 (1991) using the Mineral Powder Diffraction file of the JCPDS and by checking the inorganic Powder Diffraction File of 1991 by hand. By these means one is able to state that fraction A is composed mostly of magnetite and by minor contribution of hematite and quartz. Fraction B indicates the presence of almost pure pyrite (FeS_2) (Fig. 9) and the source of arsenic could be substitution of sulfur by arsenic within pyrite. To see whether any other arsenic-rich mineral such as the common one arsenopyrite (FeAsS) is present in the sample or not, we tried to make the known spectra of FeAsS with sample spectra (Fig. 9), but there is no FeAsS in the sample as evident from the spectra. A minute amount of rozenite ($FeSO_4.4H_2O$) is found in the spectra and that may be due to the oxidation of pyrite (Fig. 9).

Fraction C shows the dominance of quartz over minor amounts of two feldspars, albite and microcline, and also some colored minerals not visible in the spectrum due to their small concentration.

SCANNING ELECTRON MICROSCOPY STUDY (SEM)

The chemical analyses of the sediment samples E and B are shown in Table 7 which indicate the presence of high concentrations of As, Fe, Pb, Zn in these two samples. The SEM study of sample E indicates the presence of iron sulfide within a wood fragment. Sample B is notable for the presence of abundant gypsum. In one sample (62A) melantenite ($FeSO_4 \cdot H_2O$) was identified which is likely a product of recent oxidation.

TABLE 6

Mineralogy and XRD study of the arsenic-rich selected particles

Location	Site name	Mineralogy and texture	Depth (m)	Fe (%)	As (mg/l)
Katlamari, Block Raninagar-II, Murshidabad	B	Py, Qtz, Gyp; gray sand with small Py grains cemented by Gyp(?), no indication of Fe-oxide.	155–195	27	2250
Katlamari, Block Raninagar-II, Murshidabad	D	Cal, Py, Qtz; dark gray earth sand with abundant carbonized plant fragments.	137–152	21	2120
Block Kaliganj, Nadia	A	Qtz, Gth, Cal; fine orange–brown powder.	60–70	16	130
Gadigacha, Block Nabadwip, Nadia	62A	Py, Qtz, Cal(?) mica, feldspar; Py cement of detrital grains, Cal cement.	219–223	30	2290
Block Nabadwip, Nadia	T	Fe-ox cemented detrital grains; cement predates deposition.	91–100	17	149
Santipur, Nadia	E	Pyritic wood fragment; Py replacing texture of wood associated with small crystals of calcium phosphate.	80–91	13	196
Kankpul, Block Habra-I, 24 Pgs (N)	HK-1	Py, Qtz. Org. matter; few grains 'draped' with organic matter, some Py; Fe-ox(?) coating some grains.	174–177	13	116
Mahisbatan, Block Karimpur-II, Nadia	MM-12X	Py, Qtz, mica, feldspar; Py cement of detrital grains, thin red–brown coating, Cal cement.	137–140	32	2780
Mahisbatan, Block Karimpur-II, Nadia	MM-12Y	3 grains; zircon and 2 grains with sulphide minerals attached to the surface, sulfide may have originated as a vein. Preliminary observations suggest the pyrite to be cemented to the grain not part of the original fragment.	137–140	11	2170
Mahisbatan, Block Karimpur-II, Nadia	MM-12YH	Qtz, Py, mica, plagioclase; gray earthy sand 10% pyrite; 80% quartz; pyrite is coated with a gray chalky soft phase that dissolves in HCl.	137–140	6	1190
Sethpur, Barasat-I, 24 Pgs (N)	S-70	Qtz, Fe-ox, Py, Rz	209–212	35	410

Note: Py = pyrite; Qtz = quartz; Gyp = gypsum; Cal = calcite; org. = organic carbon; Gth = goethite; Fe-ox = iron oxide; Rz = rozenite.

TABLE 7

The chemical analysis of the sediment samples

	E	B
Fe_2O_3 (wt%)	30.0	32.0
As (ppm)	2600	2100
Pb (ppm)	920	1100
Zn (ppm)	500	660

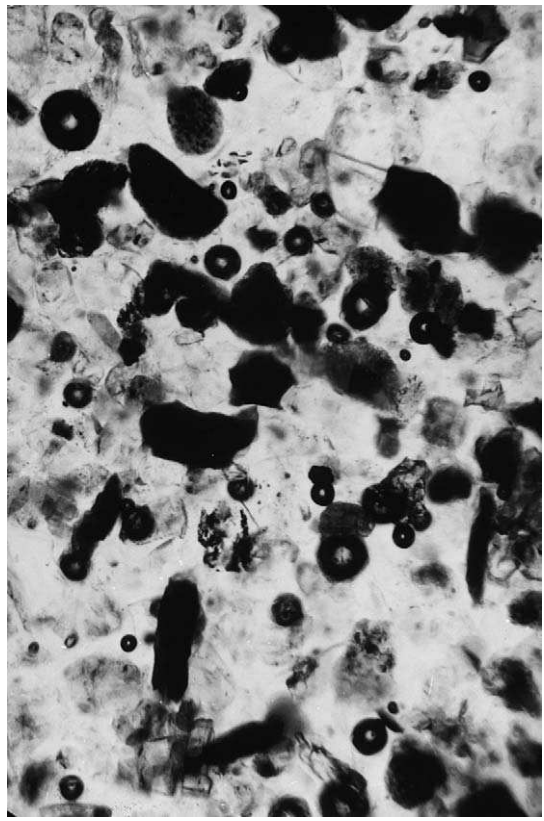


Fig. 6. Bulk core-boring sediment samples under plane-polarized light showing opaque grains.

TABLE 8

EPMA analysis of borehole sediment

Spec.	Mg	Al	Si	S	K	Ca	Ti	Fe	Zn	As
118474	0.00	1.25	0.91	46.08	0.02	0.39	0.09	51.51	0.00	0.07
118475	0.72	0.89	0.94	48.05	0.00	0.00	0.10	48.83	0.11	0.40
118476	0.00	1.00	1.20	50.89	0.00	0.03	0.11	45.91	0.15	1.36
118477	1.15	0.59	0.43	50.00	0.00	0.19	0.07	47.01	0.14	0.41
118478	0.00	0.85	0.94	52.00	0.00	0.00	0.11	45.40	0.22	0.88
118479	0.00	1.29	2.11	51.06	0.03	0.19	0.09	44.73	0.25	0.82
118480	0.00	1.18	2.06	50.84	0.00	0.11	0.10	45.15	0.13	0.67

ELECTRON PROBE MICROANALYSIS (EPMA) STUDY

An EPMA study of the selected particles B, MM12X, 62A from arsenic-rich borehole sediments (Table 6) was carried out. Seven pyrite areas (bright in Fig. 10) were analyzed from MM12X. This corresponds very closely to FeS_2 (46.5% Fe and 53.5% S) as shown in Table 8. One pyrite lump is shown in Fig. 11. The spectra of the samples B, MM12X and 62A indicate pyrite with arsenic.

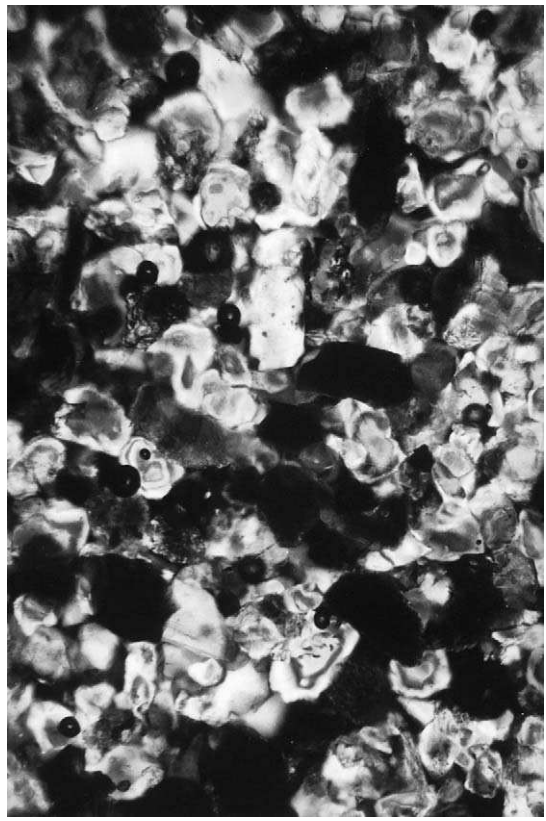


Fig. 7. Bulk core-boring samples under crossed nicols showing opaque grains.

LASER MICROPROBE MASS ANALYSIS (LAMMA) STUDY

A LAMMA study of the selected particles from sediment S-70 (Table 6) in both positive and negative modes indicates the presence of iron pyrite and arsenic in the bombarded S-70 particles. For a better understanding, a pure iron pyrite standard (Merck Chemicals, Germany) was also examined simultaneously by LAMMA in both positive and negative modes. Fig. 12 shows a comparative study of S-70 in the positive mode with pure iron pyrite of Merck. From this comparative study it appears that S-70 contains pyrite. Spectra in the negative mode also show the same similarities between S-70 and Merck pyrite. It appears that sample S-70 contains pyrite. The arsenic present in the pyrite may be either replacing sulfur or entering into its lattice structure.

ROTO-EVAPORATED ARSENIC-RICH WATER RESIDUES FROM CONTAMINATED HAND-TUBEWELLS

After studying arsenic along with other heavy metals in borehole sediment samples from arsenic-affected districts of West Bengal, it was felt necessary to study the heavy metals in the arsenic-rich underground water from hand tubewells of the affected districts. In this work, the water samples (each 10 l) were roto-evaporated and finally the solid mass obtained was dried on silica gel in a vacuum desiccator. Twenty-two well water samples having an arsenic concentration in the range of 200–2480 μg/l from eighteen affected

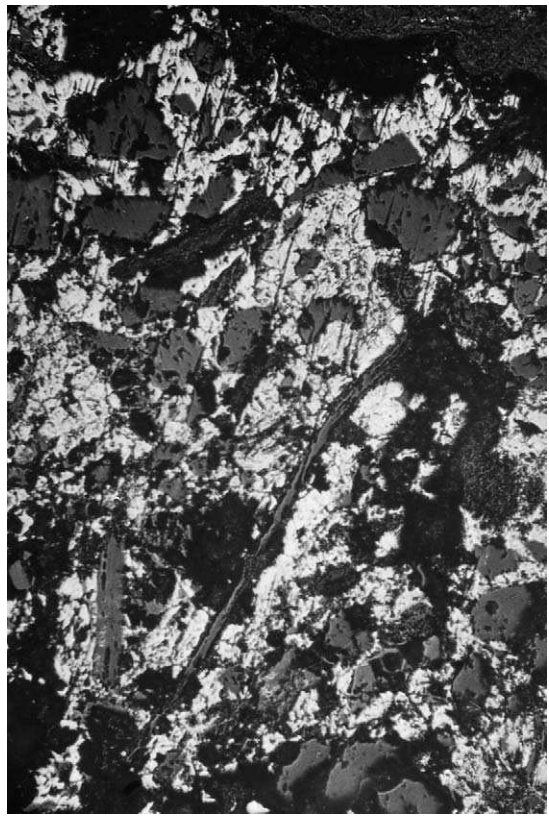


Fig. 8. Polished section study under ore microscope of some arsenic-rich particles from sludge samples showing huge amounts of pyrite grains.

blocks, were roto-evaporated to dryness. While studying the borehole sediment samples we observed good correlations in linear regression between As and Fe, Pb, Mn and Zn. Regression analyses were further carried out between the arsenic and other heavy metals for all the 22 water samples which were evaporated to dryness. The linear regression shows good correlations also between As and Fe, Pb, Mn and Zn (Table 9). The similar correlations between arsenic and heavy metals in borehole sediment and water residues (Table 9) indicates the possibility that arsenic and heavy metals in water are of sedimentary origin.

PROBABLE MECHANISM OF ARSENIC RELEASE FROM SOURCE TO AQUIFER

The identical values of the As/Fe ratios in samples of borehole sediment/core-boring/ selected particles and water roto-evaporated to dryness indicate that probably both As and Fe come from a definite source. Mineralogy, EPMA, SEM, LAMMA, and XRD studies indicate the existence of pyrite grains. Polished section studies of selected samples from sediment indicate very large numbers of pyrite grains embedded in the argillo-calcareous rock. Again, the XRD study of sample S-70 indicates the existence of rozenite ($FeS_4.4H_2O$) and also in the selected sample 62A we have identified melanferite indicating recent pyrite oxidation. Overall, these findings clearly indicate that pyrite is in the sediment. Arsenic is released most probably from the sulfide mineral due to oxidation. This could be increased by lowering the water table or increasing recharge water containing dissolved oxygen.

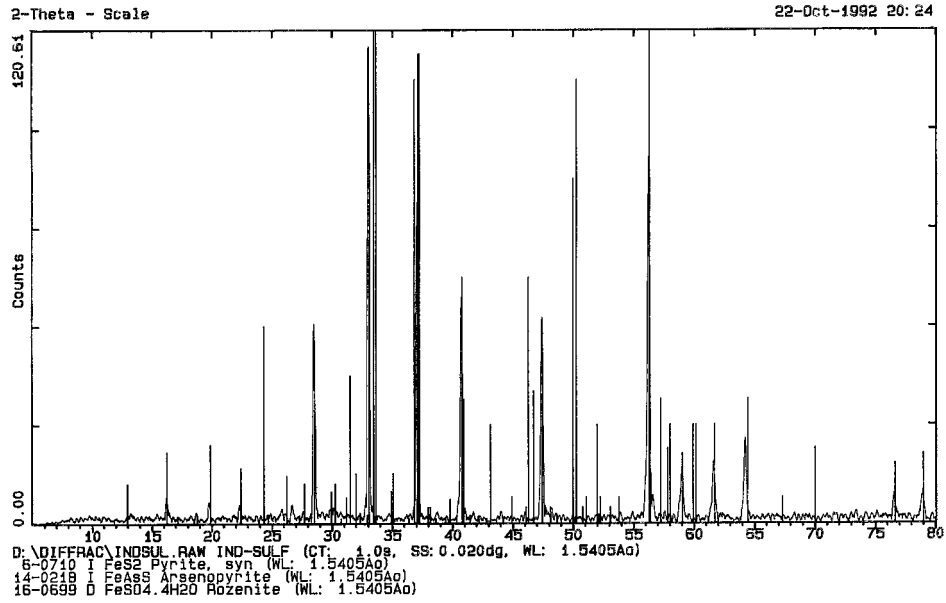


Fig. 9. X-ray diffraction study of sample S-70 indicates the presence of pyrite grains and the presence of rozenite.

Again, absorbed arsenic might have been released from iron oxy-hydroxide due to reducing conditions in the aquifer (Bhattacharya et al., 1998; Nickson et al., 1998, 2000). The iron oxy-hydroxides could be a result of previous oxidation of arsenic-rich sulfides. The overall release of arsenic from sediment could be enhanced by increased concentrations of competing ions, such as nitrate, phosphate from application of fertilizer (Acharya et al., 1999) or an increase in the pH. Aqueous nitrate in the presence of oxygen can oxidize pyrite at pH values greater than 5 (Appelo and Postma, 1993). Bacterial oxidation of Fe(II) by *Gallionella ferruginea* and sulfur in pyrite by *T. denitrificans* promotes this reaction. Pyrite oxidation involving nitrate has produced arsenic concentrations in wells above

TABLE 9

Correlation between arsenic and heavy metals in borehole sediment samples and water residues in 24-Parganas (North) district

Serial No.	Borehole sediment samples	Water residues
1	As and Fe ($r^2 = 0.99$, $P = 0.0034$) ($r^2 = 0.78$, $P =< 0.001$) ($r^2 = 0.89$, $P =< 0.001$)	As and Fe ($r^2 = 0.992$, $P = 0.0037$)
2	As and Pb ($r^2 = 0.739$, $P = 0.006$)	As and Pb ($r^2 = 0.854$, $P = 0.008$)
3	As and Mn ($r^2 = 0.98$, $P = 0.011$)	As and Mn ($r^2 = 0.872$, $P = 0.006$)
4	As and Zn ($r^2 = 0.82$, $P =< 0.001$)	As and Zn ($r^2 = 0.891$, $P = 0.016$)

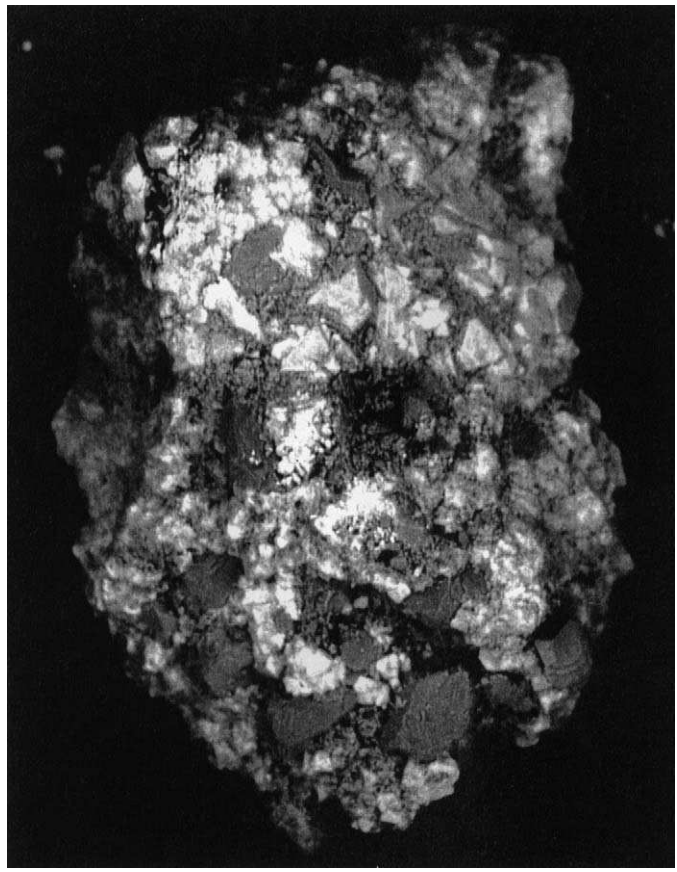


Fig. 10. EPMA analysis shows pyrite areas (bright) of sample MM12X.

50 μg/l (Van Beek et al., 1989; Appelo and Postma, 1993). If the source of arsenic is arsenic-rich pyrite in the arsenic-affected nine districts of West Bengal, then the question becomes how arsenic came into the underground sediment and how arsenic was released from pyrite. After going through all our experimental findings of borehole sediments it is felt that there may be an explanation. In the Ganges Basin there is deposition of weathered base metals. Due to reducing conditions of the delta, available iron and sulfur combined to form FeS_2 (authigenic formation) and arsenic from arsenic-rich base metals was entrapped in the crystal or replaced sulfur in pyrite. Then the question comes why and how pyrite decomposed and released arsenic. Although iron pyrite is not soluble in water, it can decompose to ferrous sulfate, ferric sulfate and sulfuric acid when exposed to air or in water containing dissolved oxygen. In these nine arsenic-affected districts millions of cubic meters of groundwater are withdrawn by big-diameter tubewells from shallow and deep aquifers for agricultural irrigation and in most cases such tubewells are not sealed with cement and concrete. As a result there is the possibility of air flowing to the aquifer (Mallick and Rajgopal, 1996). Further, the upper delta plain is mostly under unconfined conditions except near the southern fringe and there is a fluctuation of the water table which is increasing every year (Report on arsenic pollution in groundwater in West Bengal, October, 1994). Thus, groundwater is aerated. Pyrite oxidation is a complex chemical process that may involve 15 or more steps (Welch et al., 2000) resulting in an

Fig. 11. EPMA analysis shows a pyrite lump in sample B.

overall reaction commonly written as $FeS_2 + 15/4\ O_2 + 7/2\ H_2O \rightarrow Fe(O)_3 + 2H_2SO_4$. Because arsenic concentrations in pyrite generally range from 0.02% to 0.5% and may be as large as 6.5% (Kolker et al., 1998), this reaction can release a considerable amount of arsenic to the aqueous phase. The bacteria *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, and *Leptospirillum ferrooxidans* can increase reaction rates for sulfide mineral oxidation by about five orders of magnitude compared with abiotic reaction rates (Schrenk et al., 1998). *T. ferroxidans* and *L. ferroxidans* increase the oxidation rate indirectly by catalyzing the reaction between dissolved oxygen and Fe(II) to form Fe(III). The ferric iron can then oxidize pyrite, which is a much faster reaction than the reaction of pyrite directly by dissolved oxygen.

The alternative mechanism of arsenic release suggested (Bhattacharya et al., 1998; Nickson et al., 1998, 2000) is dissolution of arsenic from arsenic-rich iron oxy-hydroxide under reducing condition. We presume that formation of iron oxy-hydroxide is from decomposition of pyrite. Our borehole sediment analyses show (Fig. 2) that arsenic is present in elevated concentrations at all depths and also arsenic-rich pyrite at several depths. Also, the Asia Arsenic Network (AAN) (Akai et al., 1998) reported the existence of arsenic-rich pyrite in mud in the upper level at 3–10 m depth and also at 45–46 m; the same paper also reported the existence of framboidal pyrite. An association of arsenic with pyrite in carbonate was found by EPMA analysis (Akai et al., 1998). Existence of Jarosite in

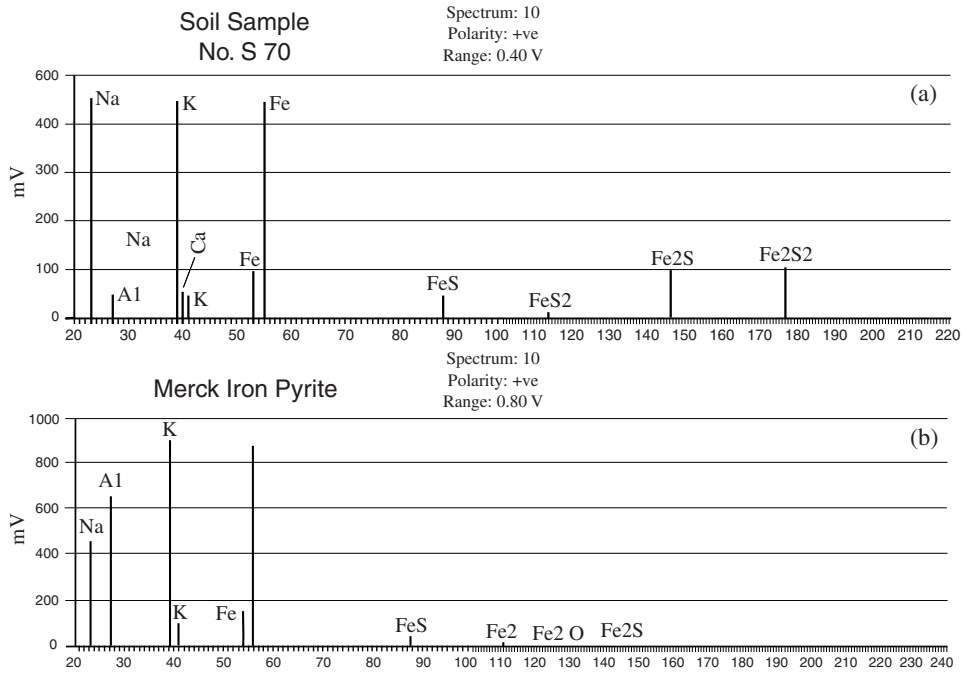
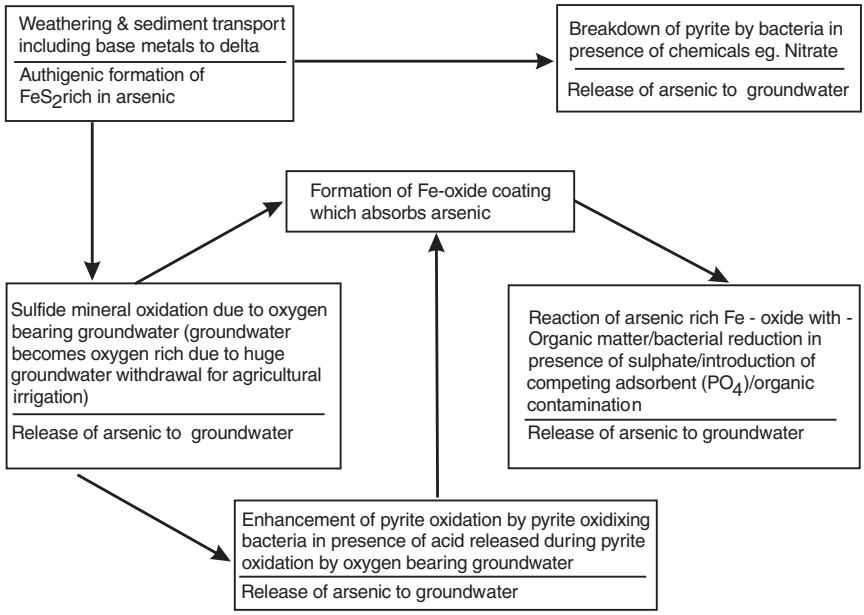


Fig. 12. (a) LAMMA study of particle S-70 in a positive mode. (b) LAMMA study of a pure iron pyrite standard (Merck Chemicals, Germany) in a positive mode.

borehole sediment at a depth of 12 m was reported from Bangladesh (Sikder, 2000). Our mineralogical study also shows the existence of rozenite and melanferite which are recent decomposition products of pyrite. We feel that from the available data the whole process can be explained as follows. Arsenic-rich pyrite in the upper sediment level and also up to that depth where oxygen can enter undergoes oxidation releasing arsenic and Fe(II), Mn(II) species which react with oxygen; then oxy-hydroxides of Fe and Mn are formed absorbing some portions of dissolved arsenic which then precipitate. Under reducing conditions the precipitated Fe and Mn oxy-hydroxides are reduced and solubilized to Fe(II) and Mn(II) species again releasing the absorbed arsenic. A portion of these two dissolved constituents in sediments can diffuse upward until they are further oxidized and reprecipitated. Another portion can also diffuse downwards and precipitate as sulfide (amorphous FeS, mackinawite, greigite, pyrite). During these dissolution/precipitation processes, elements normally associated with the oxide phase will also be solubilized and eventually they will co-precipitate with Fe and Mn hydroxides or with Fe sulfides. We found also occurring with iron and manganese, high concentrations of other metals like Ni, Pb, and Cu in borehole sediments (Table 2) and selected particles (Table 5). It is often argued that if pyrite is oxidized, the aquifer should be rich in sulfate, but it is not. Gypsum may be a product of oxidation of sulfur in the pyrite to sulfate followed by calcite common to borehole samples studied. Moreover, formation of framboidal pyrite, which has been identified by us and others (Akai et al., 1998) from Bangladesh, might explain the lack of sulfate in groundwater. A conceptual model of arsenic release in groundwater is presented in Table 10.

TABLE 10

Model of arsenic release in ground water



CONCLUSION

During our survey in arsenic-affected villages in 9 districts of West Bengal for the last 12 years, we have asked about 11,000 elderly people when they began finding the classical skin lesions among villagers. Sixty percent of the elderly people reported this to be between 1975 and 1980, 15% reported around 1970 and 25% said they could not remember. The arsenic-affected oldest man (age 72) of the village Sarderpara-Nothipota, Block Tehatta, district Nadia where we had found 80% of 300 people to have arsenical skin lesions, reported that before 1980 in his village so far as he remembered he had seen none with skin lesions but that during the last 20 years more and more people got affected. Villagers of Nothipota were drinking water from one hand tubewell dug approximately in 1970. When we first measured arsenic in their hand tubewell in 1990, the concentration was 640 μg/l.

During January 1997 we colored green 167 hand tubewells (arsenic concentration less than 10 μg/l) in the village of Dangapara, Grampanchayet-Betai, Block Tehatta, district Nadia, West Bengal and told people to drink that water. During December 1999, March 2000 and June 2000 we measured the same hand tubewells (same instrument, same procedure for collection and analysis) and found about 70% of the tubewells to have arsenic concentrations higher than 50 μg/l. Further, on 6 May 1995 we had measured 50 samples from the village of Sarberia, Block Joynagar, district South 24-Pargana and colored some tubewells green (up to 50 μg/l). We measured the same tubewells on 15 July 2000 and found that 10 shallow tubewells we colored green are now unsafe to drink from, and in most of the hand tubewells (except deep tubewells) the concentration of arsenic is much higher than what we measured 5 years before. Table 11 shows the values of arsenic in some tubewells measured on 6 May, 1995 and on 15 July, 2000. Further, during November 1991 we reported that the village of Mandra of Police Station Purbasthali of the Bardhaman district is contamin-

TABLE 11

Arsenic concentration in water in some hand tubewells measured on 6 May, 1995 and 15 July, 2000

Samples No.	Concentration of arsenic (μg/l)	
	measured on 06-05-1995	measured on 15-07-2000
J-2	50	218
J-3	<10	130
J-18	27	201
J-19	<10	277
J-22	<10	50
J-25	<10	121
J-33	<10	193
J-34	42	126
J-44	<10	159
J-50	<10	207
J-9	107	150
J-29	82	184
J-31	72	247
J-36	212	520
J-37	275	504
J-40	590	630
J-41	520	974
J-47	265	638

ated and many patients with arsenical skin lesions had been identified. However, in the nearby village of Boldedanga, tubewells were also measured and found safe to drink and there was no arsenic patient in that village. During July 1999 we heard that many people in Boledanga village are now affected. We did a survey and found that 125 people are now affected drinking from one tubewell with an arsenic content of 460 μg/l. We had analyzed the same tubewell in November 1991 and found levels < 10 μg/l. We believe that these examples indicate that with time more and more tubewells are becoming contaminated. All these findings suggest that arsenic was not present in groundwater in soluble form for thousands of years. Instead, some recent reactions are going on in underground sediment and arsenic is leaching out from arsenic-rich sources.

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Geochemical and Palaeohydrological Controls on Pollution of Groundwater by Arsenic

P. Ravenscroft, J.M. McArthur, B.A. Hoque

ABSTRACT

Reduction of iron oxyhydroxide (FeOOH) and release of its sorbed arsenic load to solution is an important mechanism by which groundwater worldwide becomes polluted with arsenic. In the Bengal Basin of Bangladesh and West Bengal (India), it is the main mechanism by which arsenic pollutes groundwater. Arsenic pollution does not arise from oxidation of sedimentary sulfides nor from ion-exchange with phosphorus derived from fertilizer (or other sources). The concentration of arsenic in the sediments of the Bengal Basin is not exceptional and the occurrence of reducing conditions alone is insufficient to explain the extreme degree and extent of arsenic pollution. Extreme pollution by arsenic occurs because biodegradation of buried peat deposits drives extreme degrees of FeOOH reduction and supplies high concentrations of arsenic to groundwater (hundreds of $\mu g\ l^{-1}$). Sources of organic matter other than peat are neither reactive enough nor abundant enough to generate the amount and degree of reduction necessary to cause such severe arsenic pollution but may account for pervasive low-level contamination ($<50\ \mu g\ l^{-1}$). The distribution of known peat basins, and their ages, correlates to some degree with the surface distribution and depth profiles of arsenic. The distribution of peat and arsenic can be related to the Late Pleistocene and Holocene evolution of the Bengal Basin. Because this evolution is controlled largely by climatic fluctuations and sea-level changes, a general model emerges for predicting the occurrence of groundwater that is naturally polluted by arsenic.

Keywords: arsenic; As; groundwater; Bangladesh; Bengal Basin; Ganges; pollution; contamination

INTRODUCTION

Throughout this article the term contamination means an unusual concentration of something whilst the term pollution means an unusual concentration that leads to environmental harm. Arsenic pollution of groundwater in the Bengal Basin was documented in 1991 (PHED, 1991) and, although overlooked by a later survey (BGS, 1992), is now known to be one of the World's worst disasters affecting humans. At least 28 million people currently drink water containing more than 50 $\mu g\ l^{-1}$ of arsenic (the current Bangladesh drinking water standard), and many more consume water with >10 $\mu g\ l^{-1}$ of arsenic (Dhar et al., 1997; Mandal et al., 1998; Ullah, 1998; DPHE, 1999, 2000; http://bicn.com/acic/, 28/10/00). The human consequence of the pollution has been described as the worst mass poisoning of a population in history (Smith et al., 2000).

Understanding the source of such severe arsenic pollution has a number of important benefits. Firstly, it helps in locating polluted aquifers and in predicting the future trends of arsenic in groundwater. Secondly, it is of great significance in formulating agricultural policy. For example, a common misconception is that pumping of groundwater for irrigation has caused arsenic pollution, with one consequence being a call for a ban on tubewell irrigation in Bangladesh. Yet irrigation has been the principal factor behind Bangladesh attaining virtual self-sufficiency in foodgrain production, and hence a great diminution in the incidence of famine. A ban on groundwater irrigation, unjustified by rigorous scientific compulsion, would have severe adverse consequences for food security, rural livelihoods and the economic future of the poor of Bangladesh. We show that irrigation has no causal relation with arsenic pollution. We also show that arsenic contamination is not caused by oxidation of sulfide minerals as water tables are lowered by pumping (Mallick and Rajagopal, 1996; Mandal et al., 1998; Roy Chowdhury et al., 1999). We dismiss, giving reasons, the suggestion (Acharyya et al., 2000) that arsenic pollution is caused by ion-exchange by phosphate from fertilizers (or any other source of phosphorus).

We confirm previous suggestions (Bhattacharya et al., 1997; Nickson et al., 1998, 2000; McArthur et al., 2001) that arsenic pollution in the Bengal Basin is a natural process whereby arsenic is released during reductive dissolution of iron oxyhydroxides, a process that also reduces the sorbed arsenate to arsenite (Zobrist et al., 2000). The reduction hypothesis (Nickson et al., 1998, 2000; McArthur et al., 2001) assigns great importance to the role of organic matter, in particular, peaty sediments, in generating anoxia in groundwater. This paper gives evidence that peat is the redox driver for reduction of FeOOH and then examines the sedimentological evidence that supports the proposition that the distribution of pollution by arsenic reflects the distribution of buried deposits of peat. In doing the above we make use of our own data, data from a survey of bacterial contamination and ammonium by Hoque (1998) and data from DPHE (1999, 2000) which are available from http://www.bgs.ac.uk/arsenic/Bangladesh.

HYDROGEOLOGY OF THE BENGAL BASIN

The Bengal Basin is one of the World's largest sedimentary basins and has accumulated a great thickness of Tertiary and Quaternary clastic sediments that were transported to the area by the Ganges–Brahmaputra–Meghna river system (Morgan and McIntire, 1959). Bangladesh occupies most of the Bengal Basin and comprises mainly alluvial and deltaic floodplains except for the small uplifted blocks of the Barind and Madhupur Tracts in the central and north-western parts of the country. On the east, Bangladesh is bounded by the Chittagong Hill Tracts and in the northeast by the Shillong plateau. The sedimentary sequence typically comprises multiple fining-upwards successions of sand, silt and clay with occasional layers of gravel at the base.

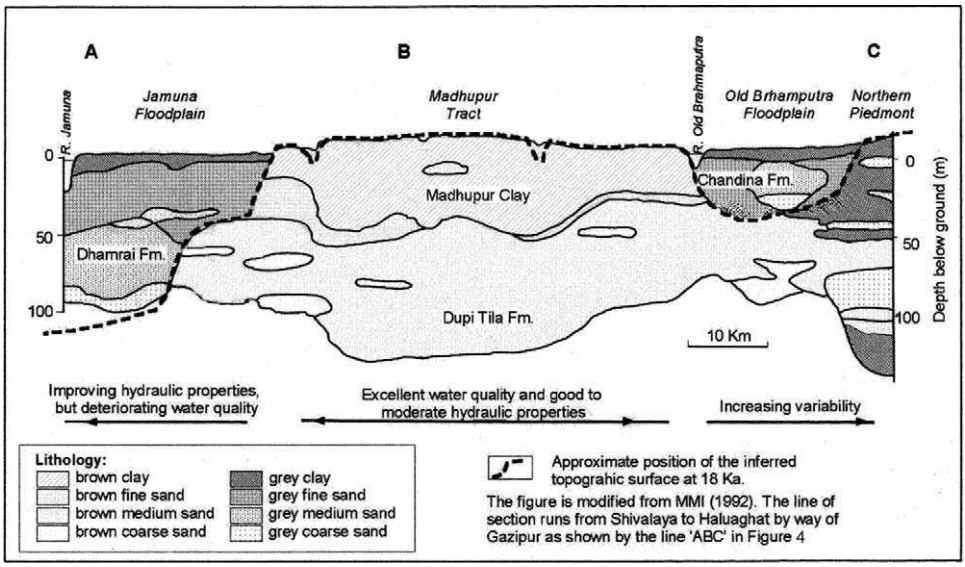

Fig. 1. Simplified geological section through NE Bangladesh. For location of section ABC, see Fig. 4.

Recent alluvium forms prolific shallow aquifers under water table or leaky conditions and, beneath the uplifted Barind and Madhupur blocks, the Plio–Pleistocene Dupi Tila Formation forms an important leaky-to-confined aquifer. Both are used for irrigation and water supply. The transmissivity of the alluvial aquifers is typically in the range of 1000–5000 m^2 day^{-1}, and is highest beneath the alluvial floodplains. The distribution of aquifer and water properties has been strongly influenced by the pattern of river incision and infilling that occurred in response to glacial–eustatic sea level changes in the Quaternary (Ravenscroft, 2001). In Fig. 1 we show a simplified geological section across north-east Bangladesh that illustrates the lateral and vertical pattern of Holocene valley-fill and Pleistocene interfluvial deposits. The characteristically brown Madhupur clay and Dupi Tila sands are the remnants of uplifted blocks that existed during sea-level low stands. Monsur (1995) deduced an early Pleistocene age for the Madhupur Clay, while Whitney et al. (1999) infer ages of 25,000 and more than 125,000 years for the surfaces of the Barind and Madhupur Tracts, respectively. The valley-fill sediments are represented by the Dhamrai and Chandina Formations. Deposits of the terminal Pleistocene–Holocene transgression reach a thickness of at least 90 m in the central Jamuna valley (JICA, 1976). However, dating of wood samples by BGS (1992) points to the presence of stepped terraces (probably Middle or Upper Pleistocene) between the central valley and the Madhupur Tract.

Sediments of the major interfluves (or *doabs* as defined by Singh et al., 1999) are brown, oxidized and highly weathered with a surface clay cover. As aquifers, they are generally confined, having low vertical and horizontal permeability and specific yields, but they contain water of excellent quality; it is typically a sodium bicarbonate type, with EC between 200 and 500 μS cm^{-1}, low iron (<1 mg l^{-1}), and with arsenic concentrations mostly less than 10 μg l^{-1}. The Holocene sediments are typically gray, unweathered, and often contain abundant organic matter. Although the Holocene aquifers have high permeability (40–80 m day^{-1}), specific yield and potential recharge rates, they have poorer water quality, the water being fresh but more mineralized (EC 500–1 200 μS cm^{-1}) and

contain higher proportions of calcium and magnesium. The waters are anoxic, high in iron, manganese and bicarbonate but mostly low in nitrate and sulfate; many are sulfidic and/or methanogenic (Ahmed et al., 1998; Nickson et al., 1998, 2000; DPHE, 1999; Gavrieli et al., 2000).

The piezometric surface is within a few meters of the surface for most of the year. In areas of intensive irrigation, water levels fall to between 5 and 15 m below ground for 2–3 months but return to the ground surface in low lying areas during the monsoon, when the aquifer is full. Only in Dhaka City, in response to intensive and continuously increasing abstraction, has the water table been permanently lowered and an original confined condition has become unconfined. Outside Dhaka City, gross annual abstraction ranges from a few tens of millimeters a year where groundwater is used only for drinking to over 500 mm a year in areas of intensive tubewell irrigation.

ARSENIC POLLUTION IN BANGLADESH

Geographical Variation

The probability of arsenic exceeding 50 and 250 $\mu g\ l^{-1}$ in the upper 150 m of the aquifer system is strongly correlated with surface geology, both in terms of age and provenance (DPHE, 1999). The distribution of arsenic-polluted groundwater beneath the floodplains of the Ganges, Brahmaputra and Meghna rivers is shown in Fig. 2, which is based on 3198 wells of depth <150 m (mostly <100 m). Summary statistics from the DPHE surveys are given in Table 1. The distribution of arsenic pollution shown in Fig. 2 differs little from that shown in Fig. 1 of McArthur et al. (2001) which was based on 19,471 DPHE field and laboratory data. The comparison indicates that the smaller sample size of the former

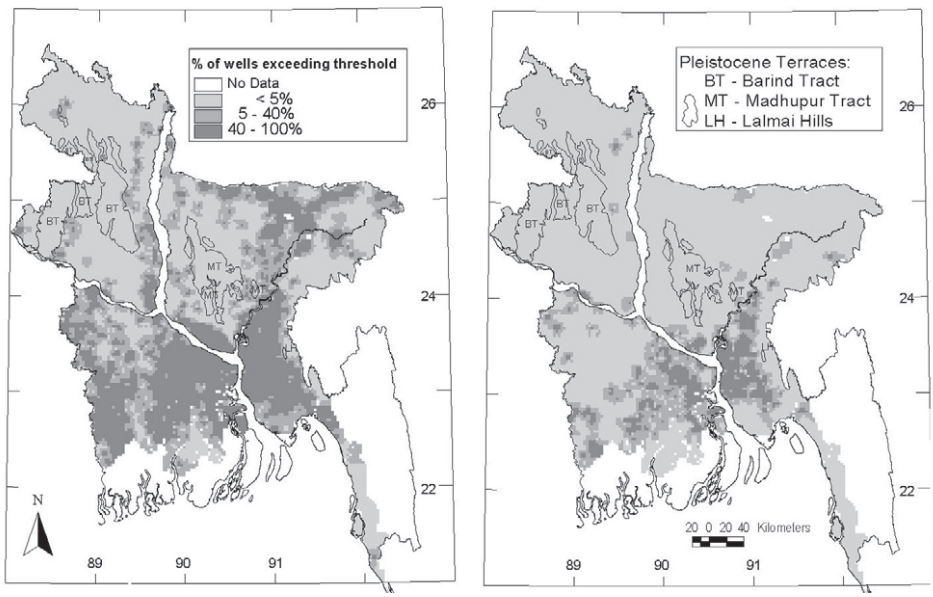

Fig. 2. Distribution of arsenic pollution in the main aquifer in Bangladesh. The interpolated surfaces show the proportion of wells exceeding (a) 50 $\mu g\ l^{-1}$, and (b) 250 $\mu g\ l^{-1}$. The surfaces were calculated using the ArcView Spatial Analyst software (based on eight nearest neighbors) from 3198 evenly spaced sampling sites from the surveys of DPHE (1999, 2000). Only wells less than 150 m deep were included in the analysis.

TABLE 1

Summary statistics, from National Arsenic Survey (DPHE, 1999, 2000)

Arsenic ($\mu g\ l^{-1}$)	No.	%	% > Lower bound
10	2042	58	–
10–50	611	17	42
50–250	639	18	75
250–500	178	5.0	93
>500	64	1.8	98
All	3534	100.0	–

is sufficient to reflect the regional (but not local) distribution of arsenic in groundwater. Except in the coastal region, the vast majority of wells are hand-pumped with short screens (mostly 3–6 m long) set at a level as shallow as practical in the aquifer. In the Lower Pleistocene and older aquifers (Barind, Madhupur and Chittagong Hill Tracts), arsenic is mostly below detection limits (around a few microgram per liter). Elsewhere, although landforms constituting the floodplains of the three major river systems differ at all scales (including village and sub-regional), aquifers underlying the Meghna floodplains are polluted most and those under the Brahmaputra floodplains are polluted least, but it is significant that arsenic pollution occurs in the catchments of all three rivers. It is also significant that the intensity of arsenic pollution increases towards the lower part of the basin (Fig. 2; Fig. 1 of McArthur et al., 2001).

Variations with Depth

Arsenic concentration is strongly dependent on depth (Karim et al., 1997; DPHE, 1999; Fig. 3). The highest concentrations of arsenic, and the highest probability of exceeding thresholds for arsenic, most often occur in wells screened between 20 and 60 m (Fig. 3) but the precise depth of the maximum varies between region and the sharpness of the concentration peak differs from place to place. In Dhaka Division (Fig. 3c), concentrations of arsenic exceed 100 $\mu g\ l^{-1}$ to depths of 100 m. In Sylhet (Fig. 3f), arsenic concentrations peak at both 60 and 120 m. The depth distributions in Fig. 3 show to be fallacious the belief that drilling deeper than 100 m provides arsenic free water; it will do so in some parts of Bangladesh, but not, for example, in the region of the Sylhet Basin. A sharp upper limit to high concentrations of arsenic appears to occur at about 10–15 m depth; few data are available for wells in the depth range 0–10 m, so this may be an artifact of data distribution. That it is not is suggested by the fact that dug wells, which are mostly much less than 10 m deep, are rarely polluted with arsenic (Chakraborti, 2001), which we presume is because they are usually oxic and so have avoided arsenic pollution from FeOOH reduction. Below 200 m, arsenic concentrations rarely exceed a few micrograms per liter (Frisbie et al., 1999; DPHE, 1999; Fig. 3).

THEORIES OF ARSENIC MOBILIZATION

Three mechanisms might explain arsenic pollution of groundwater in the Bengal Basin, viz:

(1) arsenic is released by oxidation of arsenic-bearing pyrite in the alluvial sediments (Mallick and Rajagopal, 1996; Mandal et al., 1998);

(2) arsenic anions sorbed to aquifer minerals are displaced into solution by competitive exchange of phosphate anions sourced from over-application of fertilizer to surface soils (Acharyya et al., 2000);

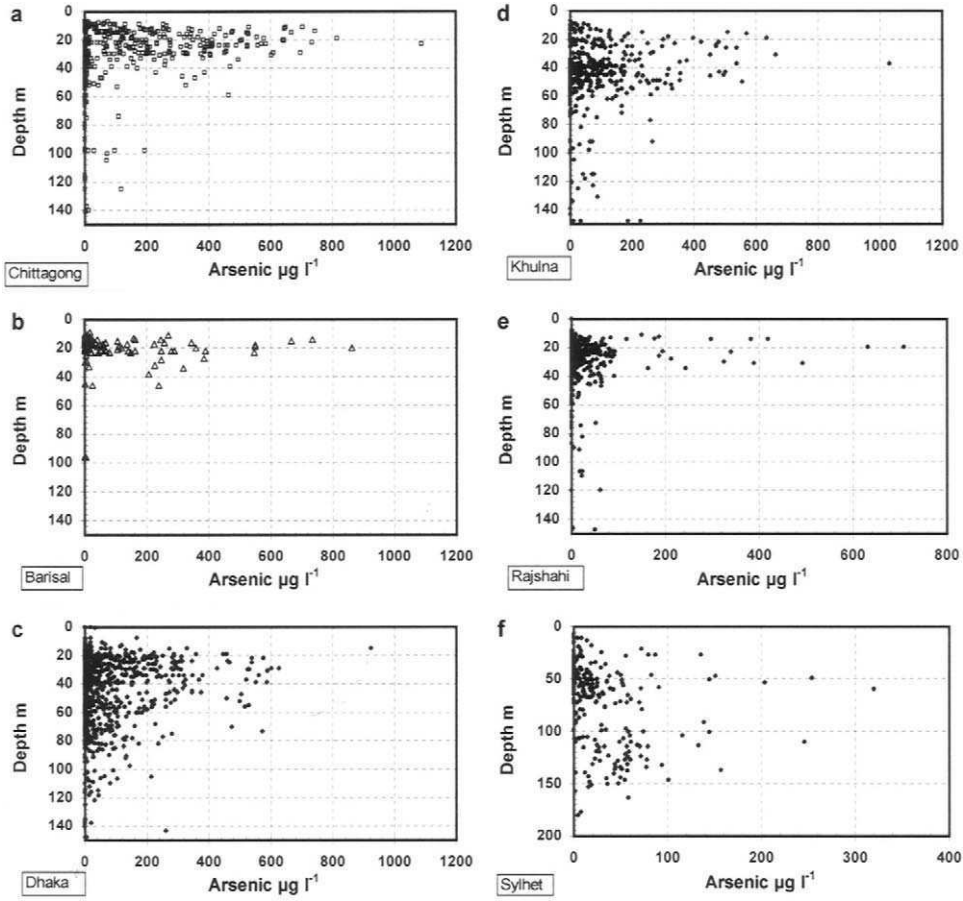

Fig. 3. Distribution of arsenic with depth in the six Divisions of Bangladesh. Depths >150 m are not plotted for a–e as wells >150 m deep seldom exceed 50 $\mu g\ l^{-1}$. Note different scales for e and f.

(3) naturally occurring arsenic that is sorbed to iron oxyhydroxides (FeOOH) is released when the iron oxyhydroxide is reduced as anoxic conditions develop during sediment burial (Bhattacharya et al., 1997; Nickson et al., 1998, 2000; McArthur et al., 2001).

Pyrite Oxidation and Irrigation Drawdown

We reject oxidation of arsenical pyrite as a mechanism for arsenic pollution in Bangladesh, even though trace pyrite is present in aquifer sediments (PHED, 1991; AAN, 1999; Nickson et al., 1998, 2000; McArthur et al., 2001). Oxidation of pyrite is envisaged to happen as irrigation draws down the water table allowing oxygen into previously anoxic aquifer sediments. A relation between the distribution of arsenic pollution (Fig. 2) and that of irrigation (Fig. 4) is therefore be expected, yet a comparison of Figs. 2 and 4 shows they bear no relation to each other: indeed, the distributions appear antipathetic. This geographical evidence does not support the existence of a causal link between arsenic pollution and tubewell irrigation. The distribution of maximum dry-season water table lowering is similar to that shown in Fig. 4 and is also antipathetic to the pattern of arsenic pollution.

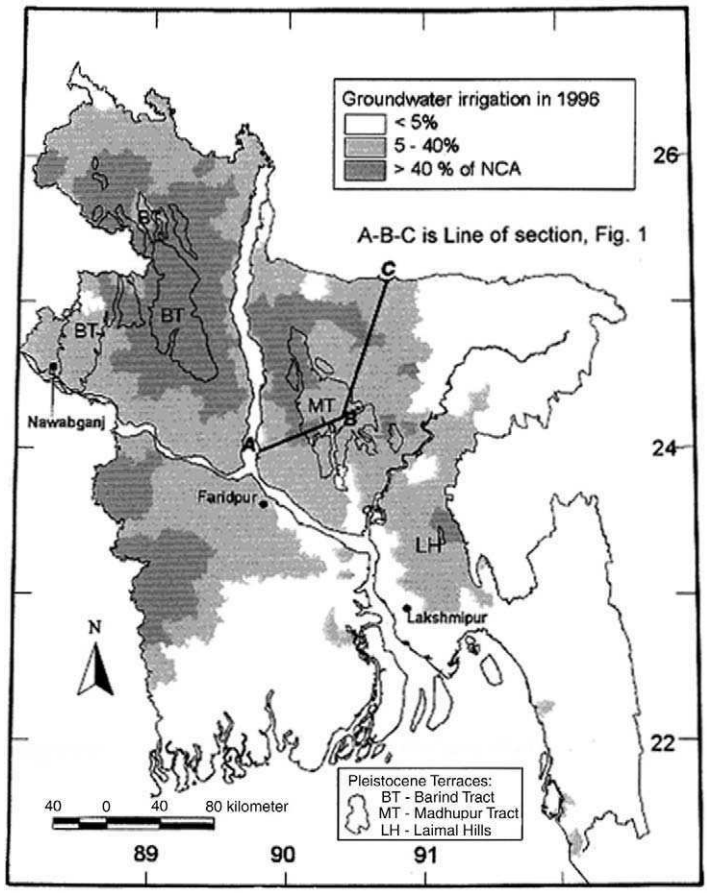

Fig. 4. Distribution of groundwater irrigation in Bangladesh in 1996. The chloropleth map shows the percentage of net cultivated area irrigated by tubewells in each upazila (an administrative unit roughly equivalent to an English or US county) in March 1996, based on the survey of NMIDP (1997).

Other reasons for rejecting pyrite oxidation as a mechanism are as follows. The sediments contain too little arsenical pyrite for this mechanism to be important. Measured sulfur concentrations in aquifer sediments represent both pyritic and organic sulfur but allow upper limits to be placed on pyrite abundance of 0.3% (Nickson et al., 2000), 0.02% (AAN, 1999), 0.1% (J.M. McArthur, unpublished) and 0.06% (DPHE, 1999). Were arsenic to be released by partial oxidation of arsenical pyrite, Bangladesh groundwaters, which are anoxic, would have an iron/sulfate molar ratio of 0.5 from the stoichiometry of the reaction:

$$2\,FeS_2 + 7\,O_2 + 2\,H_2O = 2\,Fe^{2+} + 4\,H^+ + 4\,SO_4^{2-} \quad (1)$$

In reality, iron and sulfate are mutually exclusive in solution (Fig. 5), as are arsenic and sulfate (Fig. 5); arsenic concentrations $>50\ \mu g\ l^{-1}$ occur only where sulfate concentrations are $<30\ mg\ l^{-1}$. If oxidation were complete and FeOOH was produced, rather the Fe^{2+} that would form by partial oxidation, the arsenic would be sorbed to the this FeOOH (Mok and Wai, 1994; Savage et al., 2000). Finally, arsenic pollution is uncommon in hand-dug

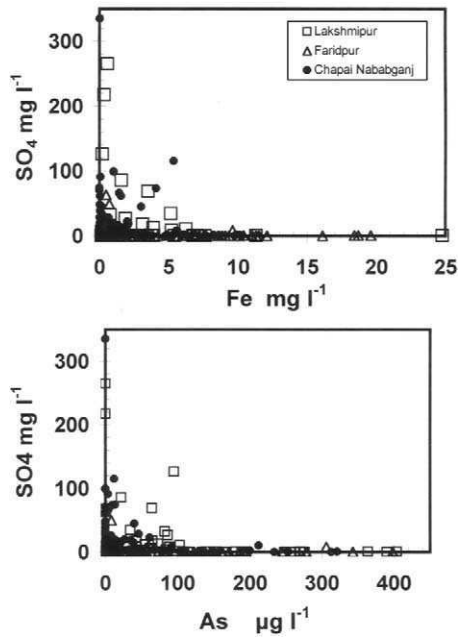

Fig. 5. Relation between iron and sulfate and between arsenic and sulfate in Bangladesh groundwaters. Data from DPHE (1999, 2000).

wells (Chakraborti, 2001) which are shallowest and most exposed to atmospheric oxygen and so would be most polluted were arsenic derived from pyrite by oxidation.

Pyrite forms during early burial, either by reaction of dissolved hydrogen sulfide (from sulfate reduction) with solid FeOOH or reaction of dissolved Fe^{2+} with dissolved sulfide ions. In the former case, arsenic sorbed on FeOOH will be incorporated into the resulting pyrite. In the latter case, as iron reduction precedes sulfate reduction in the sequence of diagenetic reactions, the arsenic released by FeOOH reduction will be sequestered from solution by pyrite forming from later sulfate reduction. The presence of trace pyrite in Bangladesh sediments shows that it has not been oxidized and so is not a source of arsenic in Bangladesh groundwater. Beneath Samta Village (Fig. 6), concentrations of arsenic and iron co-vary strongly and show a maximum within a layer of peat at about 10 m depth that acts as a reducing locus for pyrite formation; this peat layer is also a locus of arsenic removal from groundwater as arsenic is incorporated into diagenetic pyrite. Under the anoxic conditions of the groundwater, this arsenic is immobilized in pyrite and neither the pyrite in the peat nor the peat itself is a source of arsenic to groundwater.

Competitive Exchange with Fertilizer-Phosphate

The fact that the use of phosphate fertilizer in Bangladesh has increased greatly over the past 15 years has led to suggestions (e.g. Acharyya et al., 2000) that the arsenic pollution may result, at least in part, from the displacement from FeOOH of sorbed arsenic as a result of competitive (anion) exchange by phosphate leached from soils after excessive use of phosphate fertilizer. We reject this idea. The use of phosphate fertilizer in Bangladesh is widespread (Table 2) but amounts used are not high by international standards. There are extensive areas of the country where groundwater is essentially free of both arsenic and phosphorus (Figs. 2 and 7) and it is in these areas that irrigation is most intense and application of fertilizer might be expected to be highest. This argument alone seems to us sufficient to reject any link between fertilizer and arsenic. Another such argument is

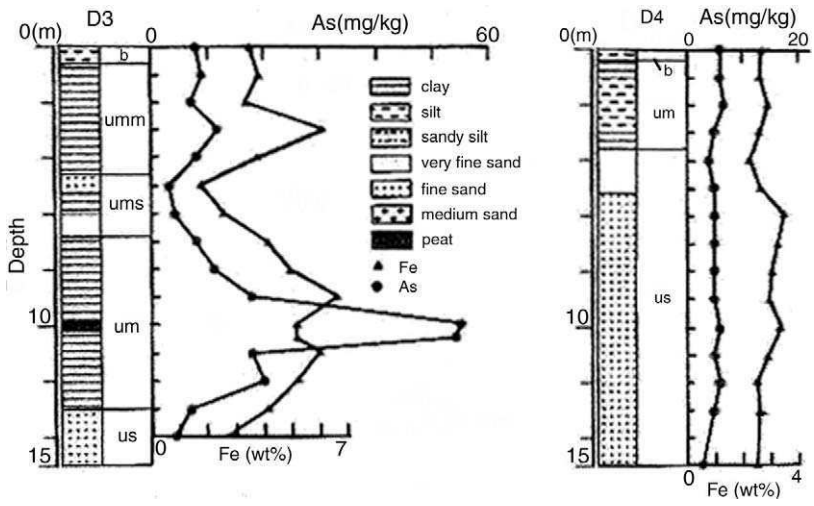

Fig. 6. Distribution of Fe and As in two sediment cores beneath Deulhi Village (D3) and Samta Village (D4), near Jessore, SW Bangladesh showing the occurrence of peat beneath the former. Reproduced from Fig. 11 of AAN (2000). Note the arsenic and iron peak where peat is present (D3) and the uniformity of both where it is not (D4). Further information on peat distribution beneath Samta and Deulhi villages, and around Mymensingh, is given by Ishiga et al. (2000).

TABLE 2

Figures typical of fertilizer use in Bangladesh

Crop	Fertilizer (kg ha^{-1})		
	Urea (46% N)	TSP (46% P_2O_5)	MOP (50% K_2O)
HYV T. Aus (irri.)	80	40	10
HYV Boro	170	70	25
HYV T. Aman (irri.)	30	110	50
HYV T. Aman (rainfed)	110	50	15

TSP = triple-superphosphate; MOP = muriate of potash. Source, National Water Management Plan project of Bangladesh.

that waters attain a bicarbonate concentration of at least 200 mg l^{-1} before phosphorus, arsenic, or iron, are found in significant amounts (McArthur et al., 2001). Waters lowest in bicarbonate are the youngest and least evolved, but they would contain most phosphorus (and so arsenic), were phosphorus supplied from surface application of fertilizer.

The fact that phosphorus in groundwater cannot contribute much to arsenic pollution is shown by the experimental desorption by phosphate of arsenic sorbed to mineral surfaces (Manning and Goldberg, 1997). These authors showed that P/As mole partition ratios for desorption of arsenic by phosphate were around 5000. Given this ratio, no more than 2 $\mu g\ l^{-1}$ of arsenic would be desorbed by a phosphorus (as P) concentration in groundwater of 5 mg l^{-1}, the approximate upper limit for Bangladesh groundwaters. Add the fact that ageing increases arsenic retention on FeOOH and it can be seen that arsenic pollution by competitive exchange with phosphorus will be negligible, whatever the sources of phosphorus.

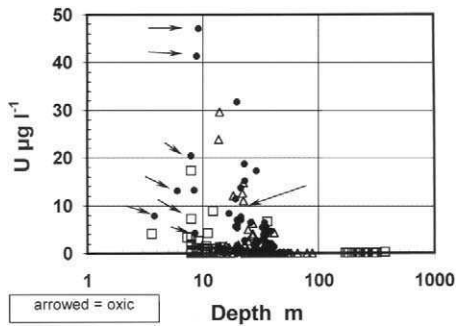

Fig. 7. Distribution of uranium with depth in Bangladesh well waters. Open squares = Lakshmipur; closed circles = Nawabganj; triangles = Faridpur. Arrows show wells reported to have measurable concentrations of dissolved oxygen.

A final argument concerns the fact that phosphate fertilizer contains uranium, so this element may be used as a tracer for fertilizer phosphate. In Bangladesh groundwater, concentrations of uranium are mostly very low; where uranium is found in high concentrations, the concentrations of phosphorus are low to zero. The distribution of uranium is therefore de-coupled from the phosphorus distribution probably because, on infiltration, fertilizer phosphorus is taken up by plants (as intended) whilst fertilizer uranium, which is mobile in the oxic environment, moves downwards to the water table and contaminates some wells. Furthermore, uranium is not found in wells greater than 41 m deep (Fig. 7), whilst phosphorus concentrations increase with depth in both Faridpur and Lakshmipur (not shown, but based on DPHE, 2000). These arguments further reinforce our case that phosphorus from fertilizer has no significant role in causing arsenic pollution. Nevertheless, concentrations of uranium are high (Fig. 7) in (mostly oxic) shallow concrete ring wells in Nawabganj, viz. BTS428 (47 $\mu g\, l^{-1}$ U) and BTS438 (41 $\mu g\, l^{-1}$ U, both 9 m); BTS347 (20 $\mu g\, l^{-1}$ U, 8 m deep); BTS 357 (U 8 $\mu g\, l^{-1}$, 4 m deep) and BTS 355 (4 $\mu g\, l^{-1}$, 9 m deep) and we cannot discount entirely the possibility that some uranium is leached from the concrete (although not all wells with high uranium are concrete ring wells).

The idea that fertilizer-phosphate has caused, or exacerbated, the arsenic problem may have arisen because groundwaters in the Bengal Basin have high (5 mg l^{-1}) concentrations of phosphorus (Frisbie et al., 1999; DPHE, 1999, 2000). This arises from release of phosphorus sorbed to FeOOH during iron reduction (minor) and from microbial degradation of organic matter, both peat and human organic waste (major; McArthur et al., 2001). Thus, this discussion breaks the link between agricultural and arsenic pollution, a break that may have a positive benefit on policy and so on the farmers of Bangladesh.

Reduction of FeOOH

Reduction of FeOOH is common in nature and has been invoked previously to explain the presence of arsenic in anoxic surface waters (Aggett and O'Brien, 1985; Cullen and Reimer, 1989; Belzile and Tessier, 1990; Ahmann et al., 1997) and anoxic groundwaters (Gulens et al., 1979; Matisoff et al., 1982; Korte, 1991; Korte and Fernando, 1991; Bhattacharya et al., 1997; Nickson et al., 1998, 2000; McArthur et al., 2001; refs. therein). Reduction of FeOOH (Eq. 2) is a microbial process

$$8\,FeOOH + CH_3COOH + 14\,H_2CO_3 \longrightarrow 8\,Fe^{2+} + 16\,HCO_3^- + 12\,H_2O \qquad (2)$$

that is driven by microbial metabolism of organic matter, particularly acetate (see Nealson, 1997; Lovley, 1997; Banfield et al., 1998; Chapelle, 2000; Lovley and Anderson, 2000) and is accompanied by microbial reduction of arsenate to arsenite (Zobrist et al., 2000, but also Dowdle et al., 1996; Ahmann et al., 1997; Stolz and Oremland, 1999). That reduction of FeOOH is common and intense in the aquifers of the Bengal Basin is shown by high concentrations of dissolved iron (24.8 mg l^{-1}, DPHE, 1999; 29.2 mg l^{-1}, Nickson et al., 2000;

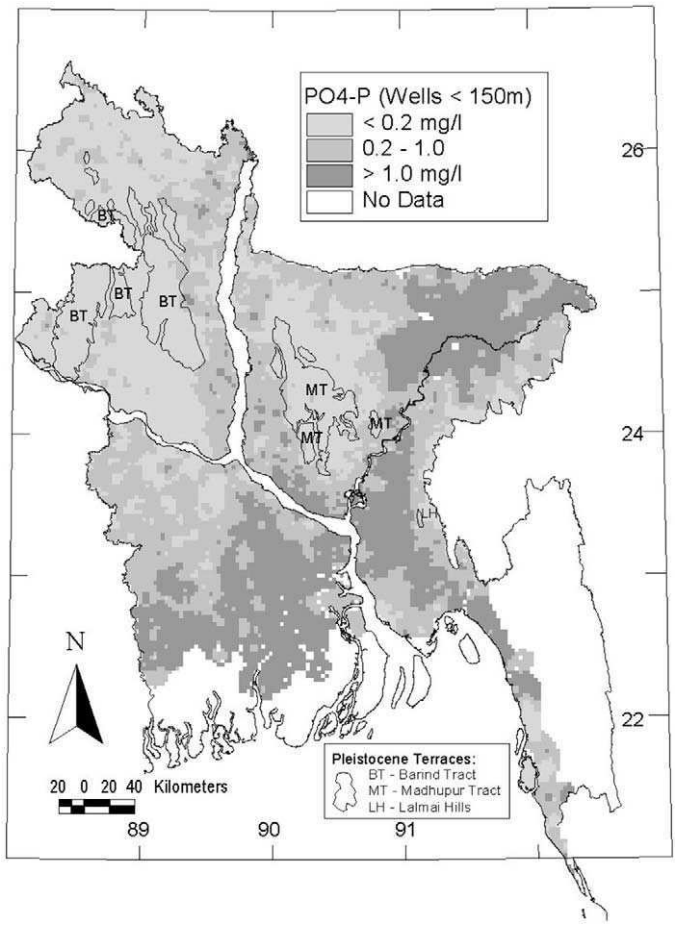

Fig. 8. Distribution of phosphorus in the main aquifer in Bangladesh. The surface of phosphorus concentration was calculated using the ArcView Spatial Analyst software (based on eight nearest neighbors) from 3198 evenly spaced sampling sites from the surveys of DPHE (1999, 2000). Only wells less than 150 m deep were included in the analysis.

80 mg l^{-1}, Safiullah, 1998); that it is accompanied by reduction of As(V) to As(III) is shown by the fact that dissolved arsenic is present overwhelmingly as arsenite (Gavrieli et al., 2000). The finding of significant amounts of arsenate in Bangladesh groundwater (DPHE, 1999, 2000) is an artifact of the storage of iron-rich waters before analysis, a procedure known not to preserve arsenic speciation (Cherry et al., 1979; Korte and Fernando, 1991; Borho and Wilderer, 1997; Hall et al., 1999; Volke and Merkel, 1999; G. Cutter, personal communication, 2000). Given that microbial metabolization of organic matter drives reduction of iron and arsenic, the generation of some organic arsenic species might be expected. Using the method of Wangkarn and Pergantis (2000), we have analyzed for MMAA and DMAA in water from 13 wells in Faridpur, sampling in both February 2000 and March 2001. In no sample was either above the detection limit of 2 ppb.

The spatial distribution of phosphorus in well-water closely parallels that of arsenic (cf. Figs. 2 and 8), although the well-by-well correlation of arsenic and phosphorus concentra-

tions is modest. This similarity of distributions points to a common diagenetic origin for arsenic and phosphorus. Both elements form anions that sorb strongly to iron oxyhydroxide and their joint release during reduction of FeOOH probably contributes to their similar geographical distributions.

An invocation of FeOOH reduction to explain arsenic pollution needs to address why some groundwaters in Bangladesh are high in iron and low in arsenic and others are low in both. Arsenic pollution and iron are absent from groundwaters in aquifers of Lower Pleistocene and older age, beneath the Madhupur Tract and the Barind Tract, because these aquifers are oxic and, particularly where iron has been recrystallized as more stable phases such as hematite (BADC, 1982), reductive dissolution of iron oxyhydroxides does not occur in them. In coastal Lakshmipur, where both shallow (<40 m) and deep (>130 m) aquifers are anoxic and contain dissolved iron, only the shallow aquifers contains pollutant arsenic. It may be that arsenic in the deep aquifer was mobilized during early diagenesis but has long since been flushed out by meteoric waters, which have been flushing the deep aquifer since well before the 18 ka sea level minimum of around 130 m below the present level. In contrast, the Holocene shallow aquifer, largely less than 7 ka in age, has not been so well flushed. Reduction of FeOOH will dissolve most of the surface-sorbed As(V) but not necessarily all the FeOOH, some of which may remain for later reduction. The As(V) will be reduced during FeOOH reduction (Zobrist et al., 2000) but not strongly re-sorbed, as As(III) sorbs less strongly to FeOOH than does As(III). A further reason why the deep aquifer is not arsenic polluted may be that sulfate reduction leads to sequestration of both iron and arsenic in diagenetic iron sulfides in the sediments, but not necessarily in the proportion in which they are seen in solution. At Lakshmipur, sulfate consumption (i.e. sulfate calculated from chlorinity minus measured sulfate), and so pyrite formation, is greater in the deep aquifer than in the shallow aquifer, so the deep aquifer has formed more pyrite and so sequestered more of its arsenic in diagenetic iron sulfides, which is not surprising given its much greater age.

An invocation of FeOOH reduction to explain arsenic pollution also needs to explain why there is seldom a good relation in groundwater between arsenic and iron, or between iron and bicarbonate. The co-variance between iron and bicarbonate (expected from Eq. 2) may be poor because bicarbonate is produced by reduction of nitrate and sulfate, and weathering reactions (driven by methanogenic-CO_2), not by iron reduction alone. Poor correlations of arsenic with iron may arise because of resorption (partial, as arsenite sorbs less well than does arsenate) of arsenic onto fresh FeOOH exposed by dissolution, or because the As/FeOOH ratio may vary from place to place, for example in response to differing amounts of FeOOH on mineral surfaces and differing mineral abundances. Finally, poor correlations between iron and other constituents may result from the sampling protocol used, in both the regional survey of arsenic pollution in Bangladesh (Volume S2 of DPHE, 1999) and the more detailed survey of groundwater chemistry in Nawabganj, Faridpur and Lakshmipur (S4 of DPHE, 1999), which required that samples be filtered at the well head. Filtering reduces the amount of arsenic in well water (D. Chatterjee, personal communication, 1999) presumably because, as previous work in Bangladesh had shown (BGS, 1992), filtering iron-rich waters may remove iron from solution unless done under anoxic conditions in a glove bag, a technique not used in any survey in Bangladesh. More than 50% of dissolved iron may be lost at low (1–2 ppm) concentrations. At higher concentrations, losses may be proportionately smaller but they may still be significant in terms of mass. The iron oxyhydroxide precipitated on the filter may scavenge arsenic during filtration. Rural consumers do not filter well-water before use so the data for the regional survey may underestimate arsenic concentrations in waters and so the risk associated with water use. Estimates of the prevalence of arsenic-related health problems that are based on the DPHE data (e.g. Yu et al., 2000) should be viewed with this potential problem in mind.

THE REDOX DRIVER

The lateral and vertical differences in arsenic concentration in well water (Fig. 3; see also McArthur et al., 2001) do not arise simply from variations in the abundance of arsenic in aquifer sediments, since sediments in the Bengal Basin are not unusual in the amount of arsenic they contain. Concentrations of arsenic exceed the low tens of ppm only where traces of early diagenetic, arsenical pyrite occur where organic matter is abundant (e.g. at 10 m beneath Samta Village arsenic peaks at 60 mg kg^{-1} where peat is found; Fig. 6). It is our postulate that pollutant arsenic in groundwater occurs where microbial reduction of FeOOH has released sorbed arsenic (and also reduced it) and organic matter is required to drive the process. We postulate that it is the distribution of organic matter in the aquifer sediments that is the main control on the distribution of arsenic pollution and this organic matter (OM) may take several forms.

Disseminated organic matter

Concentrations of <0.5% total organic carbon (TOC) are typical of fluvial sands in the Bengal Basin (Ahmed et al., 1998; Nickson et al., 1998; AAN, 1999; DPHE, 1999) with a few higher values (e.g. 6% in Nickson et al., 1998, 2000) being found in organic-rich horizons that are discussed in a later section. Before being incorporated in the sediment, OM disseminated through the sands will have undergone some exposure to oxic degradation during riverine transport. Such exposure will have degraded the most reactive components of the OM, leaving it cellulose-rich and unfavorable for bacterial metabolization. The amount, and probably the type, of OM combine to make it a source inadequate to drive redox to the extremes seen in the aquifer, which has water with up to 80 mg l^{-1} of iron (Safiullah, 1998) and up to 24 mg l^{-1} of ammonium ion (DPHE, 2000). A negligible contribution to FeOOH reduction, and to arsenic concentrations, is expected from this source.

Organic matter in silts and clays in overbank sediments

Overbank silts and (subordinate) clays contain more organic matter (0.3–1.5%) than do fluvial channel sands (Ullah, 1998; Ravenscroft and Ahmed, 1998) leading the latter authors to suggest that arsenic pollution might be present where buried overbank deposits occur and not where palaeo-active channels are found, because the former contain more organic matter than do the latter. Whether this is sufficient OM to drive the intense iron reduction, and severe arsenic pollution, in the Bengal Basin is uncertain, but we hypothesize that it is unlikely. More importantly, the clays in overbank deposits are the least permeable strata in the sedimentary system and act as aquitards, so the availability of its OM for reaction is limited by the low permeability of such units. For this reason, and other reasons given below, we believe that such sediments are not the major redox driver for the severe arsenic pollution, although they will contribute background amounts of arsenic.

Peat Beds

Peat is common beneath the Old Meghna Estuarine Floodplain in Greater Comilla (Ahmed et al., 1998), Sylhet, and the Gopalganj-Khulna Peat Basins (Reimann, 1993; Brammer, 1996). Many wells in the area around Faridpur may be screened in waterlogged peat (Safiullah, 1998) and the shallow aquifer system in Lakshmipur contains peat (DPHE, 1999). Peat is often found in geotechnical borings (piston samples), although it is rarely recorded during rotary drilling for water wells because such drilling masks its presence unless the peat is very thick. Peat occurs extensively beneath the arsenic-affected areas of southern Samta Village and Deulhi Village in southwestern Bangladesh at a depth of about 10 m (Fig. 6; Figs. 11 and p12 of AAN, 2000; Ishiga et al., 2000). Peat has been found in Holocene sediments around Mymensingh (Ishiga et al., 2000). A further indicator of peaty sediment is the TOC content of some aquifer sediments; a sample from a depth

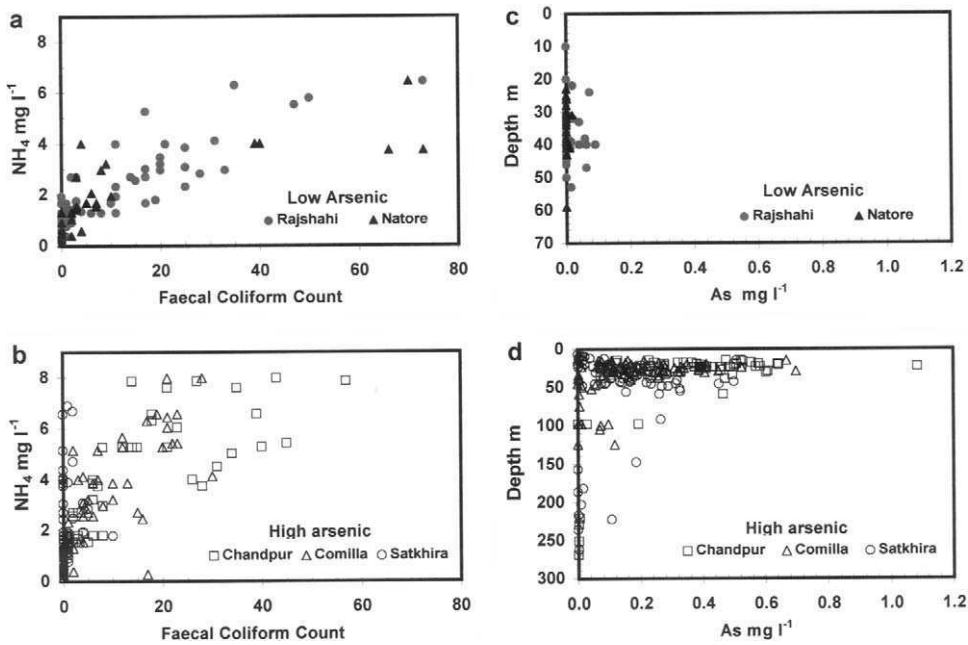

Fig. 9. Relation of ammonium and faecal coliform counts in Bangladesh groundwaters that are arsenic free (a) and arsenic polluted (b) and the relation of arsenic with depth in the same arsenic-free districts (c) and arsenic-polluted districts (d). Data from Hoque (1998).

of 2.1 m at Gopalganj (100 km SW of Dhaka) contained 6% TOC (Nickson et al., 1998); sediment from a depth of 23 m at Tepakhola (Faridpur municipality) contained 7.8% TOC (Safiullah, 1998). Peat is repeatedly mentioned by Umitsu (1987, 1993) and by Goodbred and Kuehl (2000) as being present in Bangladesh sediments. A further indicator of the degradation of much organic matter is the presence of ammonium in wells in concentrations of 24 mg l^{-1} (Hoque, 1998; DPHE, 2000) and the strong correlation between ammonium and phosphorus in well waters (McArthur et al., 2001). In addition, biogenic methane is common in groundwater over large areas (Ahmed et al., 1998; Hoque et al., 2001), in places in amounts sufficient to provide domestic fuel and to prevent suction-mode motorized pumping of groundwater; such methane can occur in the shallow aquifer only where substantial amounts of organic matter are undergoing microbial degradation. Through a combination of poverty and local pragmatism, many wells are completed with short screens at the top of the uppermost aquifer, in many cases but a short distance below a peat horizon. Were the wells deeper or the screens longer, the adverse impact on health would be avoided, or much reduced. If our arguments are correct, the areal distribution of arsenic pollution should correspond closely to the areal distribution of buried peat, so we explore this relation in the next section.

Whilst the indicators ammonium, phosphorus and methane show that biodegradation of peat is a major redox driver for arsenic pollution, a contribution to all of these pollutants occurs from degrading human organic waste in latrines, which are often sited within a few feet of wells. In areas where arsenic pollution is uncommon (Districts of Rajshahi and Natore) ammonium concentrations in wells correlates well with coliform counts (Fig. 9a). Where arsenic pollution is intense (Fig. 9b; Districts of Chandpur and Comilla) the correlation is poorer, and ammonium appears in excess, with wells lacking fecal coliforms con-

taining up to 6.6 mg l^{-1} of ammonium (Hoque, 1998), presumably because of the added contribution from degrading peat. On the basis of this comparison, and the fact that arsenic pollution would be seen more in the northwest of Bangladesh if latrine pollution was a major redox driver of FeOOH reduction, we conclude that peat degradation is the main redox driver of arsenic pollution. We nevertheless feel that the local influence of latrines requires further investigation since the siting of some wells near latrines may bias regional survey data by introducing a local noise on the regional signal of arsenic pollution.

The geographic distribution of arsenic pollution shows some concordance with the distribution of paludal basins (Fig. 10) recorded by Goodbred and Kuehl (2000). Peat deposits are, and were, formed in waterlogged areas, rather than active river-channel deposits, a fact that helps to define today's areal pattern of pollution. Umitsu (1987, 1993) proposed that much peatland development occurred in the Bengal Basin during a climatic/sea-level optimum some 5000 years BP. The high number of polluted wells with depths of between 20 and 60 m may result from their being screened close to such peat horizons. The widespread occurrence of peat layers at shallow depth has been noted by Brammer (1996).

If degradation of peat drives the redox processes that mobilize arsenic, then it should be possible to relate the distribution of arsenic to the transient palaeohydrological conditions, driven by Quaternary climatic changes, that controlled the formation of the paludal basins and related flood basins and mangrove swamps. Late Quaternary sea level changes were a major determiner of the distribution and hydrogeological properties of sediments in the Bengal Basin (BADC, 1992; Umitsu, 1993; Goodbred and Kuehl, 1999, 2000; Ravenscroft, 2001). When global sea level was 130 m lower than now at 18 ka, the main rivers were deeply incised. The proto-Ganges and proto-Brahmaputra flowed within a series of stepped terraces between the Madhupur and Barind Tracts and Eastern Hills before flowing across a coastal plain some 50–70 m below present ground level. Incision of the major rivers probably followed their modern alignments, with the exception of the Lower Ganges which took a more direct route to the Swatch of No Ground (Umitsu, 1993; Goodbred and Kuehl, 2000). The coastline then would have been far to the south of its present position. Monsoonal circulation was suppressed and rainfall reduced (Dawson, 1992). Consequently, the regional water table would have been many tens of meters below ground. Hence in sediments that have survived the maximum Pleistocene incision, the consequent oxidation depleted organic matter and promoted recrystallization of iron phases. Aquifers formed from these sediments (Dupi Tila sands) are now oxic and so mostly free of arsenic pollution.

During the initial period of post-glacial sea level rise, alluvial aggradation was largely confined to the incised axial courses of the main rivers with most sediment being transferred to the submarine delta (Kudrass et al., 1999). At about 11 ka, sea level intersected the coastal plain and wide estuaries extended deep into the major river valleys (BADC, 1992; Hoque et al., 2001). Incomplete flushing of these sediments accounts for the occurrence of residual salinity (TDS < 12,000 ppm) in fine grained strata (BADC, 1992). Between 11 and 7 ka, onshore accumulation dominated as prograding deltas built out into a steadily rising sea level in the Bay of Bengal. Goodbred and Kuehl (1999) estimate that, during the mid-Holocene climatic optimum, the average discharge of the Ganges and Brahmaputra was 2.5 times as large as it is today. A flooded coastal platform, high discharge and elevated temperature created ideal conditions for the accumulation of extensive peat deposits. Since sea level rise was simultaneous with delta progradation, it is expected that the present depths of these paludal deposits increases inland.

Based on radiocarbon dating of OM from cored boreholes across southern Bangladesh, plus secondary sources, Goodbred and Kuehl (2000) have mapped the approximate position of a series of paludal basins developed at 9, 7, 5 and 3 ka during the Holocene transgression (Fig. 10). The maps shows few paludal basins in south-east Bangladesh,

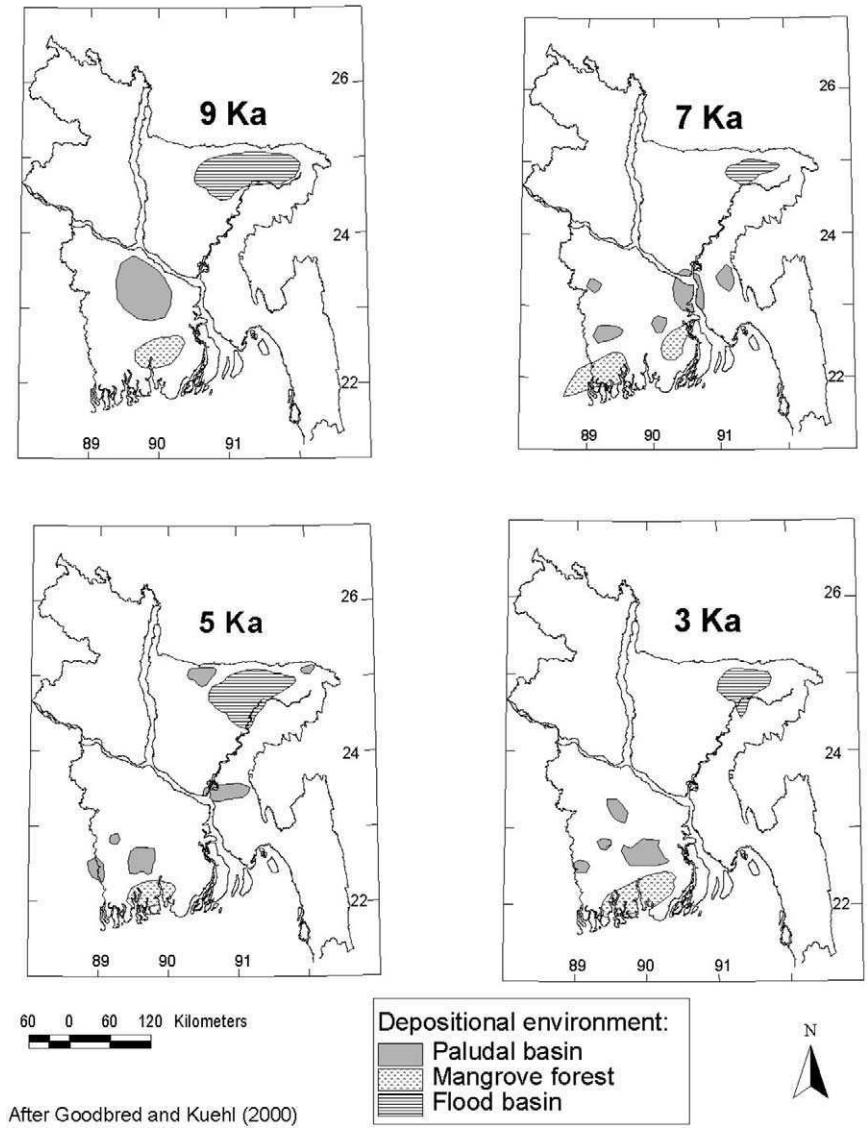

Fig. 10. Palaeogeographic map of the Bengal Basin showing location of paludal basins (after Goodbred and Kuehl, 1999, 2000).

however, we believe that buried peat is more extensive here than implied by the map because, firstly many drillers report encountering peat in the region and, secondly, there are widespread discharges there of biogenic methane in shallow wells (BADC, 1992). Ahmed et al. (1998) showed that methane is spatially associated with Na-Cl groundwater, but is released at a shallower level in the aquifer system. This pattern is consistent with paludal basins building out into a retreating Holocene estuary. Further, peat and wood (mangrove?) samples have been encountered in deeper (70–130 m) drillings across the coastal

TABLE 3

Arsenic contamination in areas of former paludal basins

Age of basin *	No. of Wells	% of wells >50 $\mu g\, l^{-1}$ As	% of wells >250 $\mu g\, l^{-1}$ As	Av. $\log_{10}$(As) $\mu g\, l^{-1}$
3 ka	77	45	16	0.033
5 ka	82	68	34	0.106
7 ka	160	62	23	0.060
9 ka	211	63	18	0.054

* Ages from Goodbred and Kuehl (2000).

plain and dated at between 30 and 40 ka (J.W. Whitney, personal communication, 1999) and so indicate the presence of a low-permeability, OM-rich, confining layer over the deep aquifer during the period of maximum incision at 18 ka.

To test the idea that high arsenic concentrations are associated with paludal sedimentation, the locations of paludal basins from Goodbred and Kuehl (2000) were superimposed upon the georeferenced arsenic analyses from DPHE (1999) and ammonium analyses from the same wells by Hoque (1998). Table 3 shows that the percentage of wells overlying the Holocene paludal basins polluted with arsenic is two to three times the national average. The proportion of polluted wells is least beneath the 3-ka basins. Beneath the older basins, the proportions of polluted (>50 $\mu g\, l^{-1}$) wells are higher but similar to each other, however, the proportion of wells exceeding 250 $\mu g\, l^{-1}$ (or as mean concentration) is much more beneath the mid-Holocene (5 ka) basins, a distribution that may indicate multiple peat horizons in this rapidly subsiding basin.

During the early Holocene, sea level rose more or less continuously until about 6 ka and then stabilized whilst perhaps being a little higher during the climatic optimum at about 5 ka. Therefore peat formed before 7 ka should be more deeply buried, and if the peat-degradation hypothesis is correct this should be reflected in deeper peak of arsenic pollution. Fig. 11 shows the depth-profiles of arsenic represented as proportion of wells exceeding 250 $\mu g\, l^{-1}$ in each 10-m depth-slice, and of the average ammonium concentration. Given the uncertainty of mapping the locations of former paludal basins, only general trends should be expected, particularly since some of the basins locations overlap and would produce complex profiles. Furthermore, the ammonium that may be derived from decomposition of peat must be judged against a background of widespread fecal contamination. Nevertheless, there are clear signatures of the 3- and 9-ka basins in both the arsenic and the ammonium profiles. The 3-ka profiles peak at a shallow level and decline rapidly with depth. The 9-ka profiles have distinct peak that is clearly deeper than in the other profiles. The 5- and 7-ka profiles are similar to each other, which is not surprising since sea-level rise leveled off at about 6 ka. The coincidence of peak ammonium and arsenic concentrations both with each other and with the increasing age of paludal deposit is therefore consistent with our proposal that decomposition of peat is the critical redox driver leading to arsenic mobilization. In Sylhet, arsenic concentrations appear to peak at 60 m and also at 120 m, a distribution that may indicate major peat horizons at two distinct levels in this area of rapid subsidence.

PHYSICAL PROCESSES OF ARSENIC MIGRATION

Biodegradation of peat releases short-chain carboxylic acids and methylated amines that will drive FeOOH reduction and ammonium production (Bergman et al., 1999). The fact that arsenic concentrations are so strongly dependent on depth (Fig. 3) suggests that

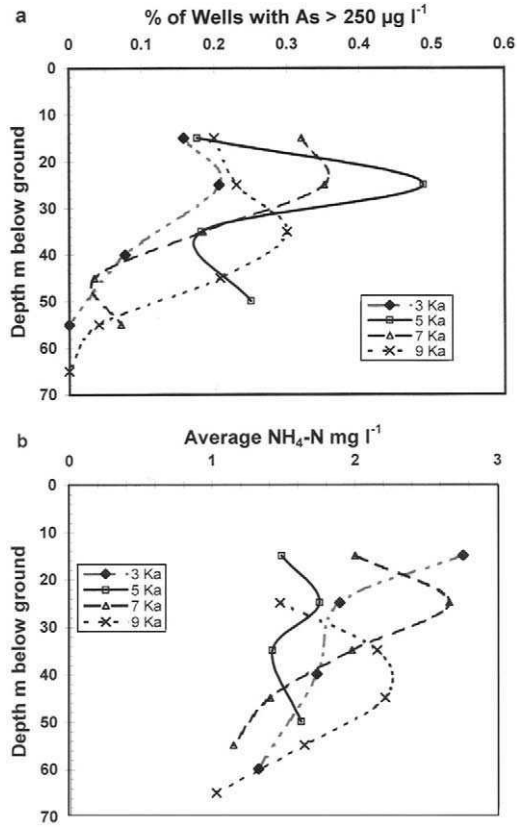

Fig. 11. Profiles with depth of arsenic and ammonium-N concentrations in paludal basins. Wells were classified into 10 m (drilled) depth intervals for statistical processing. In order to minimize the problem of unrepresentative sample sizes and extreme concentrations, only depth intervals with more than 10 water samples were used for plotting depth-profiles.

these small organic molecules have not succeeded in migrating far, in a vertical sense, from their peat source. The extremely sharp upper limit at which arsenic pollution occurs (close to 10 m depth in most areas; Fig. 3) must define the maximum depth to which recharging water carries dissolved oxygen, which will prevent redox processes except oxic degradation. The asymmetry of the profiles may result from recharge passing through shallow peat layers and spreading through the aquifer sands the small organic molecules necessary to drive FeOOH reduction and arsenic release in the aquifer (where water is captured by wells with short screens placed at the top of the aquifer). This degree of asymmetry in depth-distribution, and the depth of penetration by OM and so arsenic, will be governed by the vertical permeability distribution and by the depth of wells in a region. A difficulty with this postulate is that modern peats are typically underlain by impermeable clays (Brammer, personal communication, 2001) and so older peats may also be so underlain, thereby restricting downward migration of organic moieties; later channels may cut through peat deposits as rivers alter course and so provide pathways through channel sands for dissolved organic material to move downwards (Brammer, personal communication, 2001). A schematic of the process of how arsenic pollution affects individual wells is shown in Fig. 12.

If degradation of peat drives the reduction process, dissolved organic matter has migrated into aquifer sands that are adjacent to the peat horizons and there caused arsenic pollution of water wells. But pollution is very patchy within an area because migration

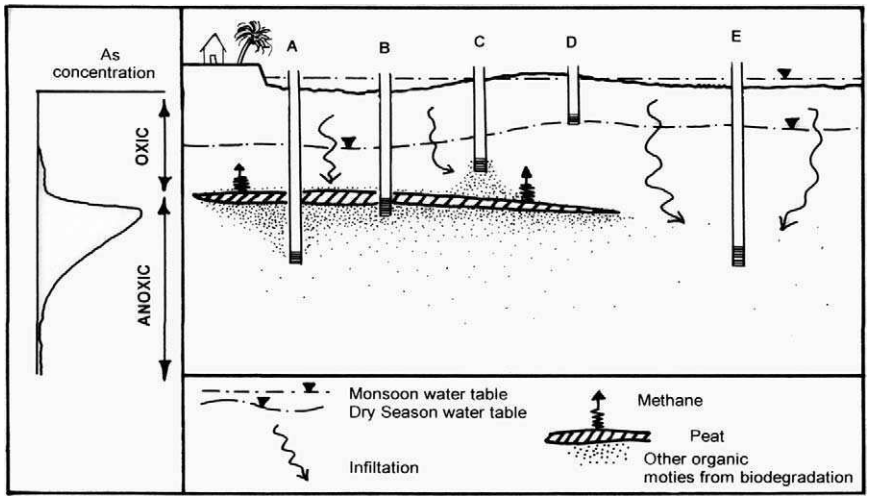

Fig. 12. Model of how arsenic pollution occurs in the Bengal Basin, and in any sedimentary sequence hosting buried swampland and marsh. In shallow Bangladesh sequences, hydraulic gradients cause downward movement of water. In deeply buried sequences (e.g. Hungary) hydraulic gradients may cause upward flow of water through peat. (A) Low concentrations of organic moieties from distant peat cause some FeOOH reduction, the release of small amounts of arsenic, and so low arsenic concentrations. (B) High amounts of organic moieties from nearby peat cause much FeOOH reduction, the release of large amounts of arsenic, and so high arsenic concentrations. (C) Organic moieties being drawn upwards by strong local pumping to cause FeOOH reduction, arsenic release, and so arsenic contamination above a peat layer. (D) Uncontaminated, oxic, hand-dug well. Seasonally dry, it is safe from arsenic pollution. (E) A well that is currently uncontaminated. The likelihood of contamination depends on the distance organic moieties travel laterally before being completely consumed by redox reactions, and on the rate of movement of dissolved arsenic.

has not spread arsenic or organic reaction products throughout the aquifer. The shallow groundwater flow system in Bangladesh extremely complex (van Wonderen, 2001). Secondary migration (through local and regional circulation) is driven by the interaction of low regional hydraulic gradients, micro-topographic effects, surface water bodies, deep-rooted vegetation, seasonal flooding and waterlogging. Human intervention (pumping and land use) further complicates these processes by inducing seasonally alternating vertical and horizontal components of flow that are superimposed on topographic gradients that operate on different and sometimes opposing directions. These processes result in a small aquifer throughflow measured on a scale of kilometers, but a rapid turnover of water in the upper 5–10 m (including the 3–5-m zone of water table fluctuation). Furthermore, small scale circulation of water and solutes in the underlying zone — a few meters to a few tens of meters thick — will be significant thereby providing a mechanism for redistributing small organic molecules and arsenic from low- to high-permeability zones

DPHE (1999) modelled the effects of (modern) natural fluctuations and flow (i.e., no pumping) on the movement of arsenic away from low-permeability (0.01–0.1 m day^{-1}) 'hot-spots' within fine sand (10 m day^{-1}) under conditions based on experience at Faridpur. Simulations were run for a 500-year period and included advection and dispersion with and without the effects of sorption. Without sorption, lateral spreads of up to 200–300 m and vertical spreads of 10–50 m were indicated. Including sorption reduces the predicted lateral and vertical spreads to around 25 m and <10 m, respectively. Thus it seems feasible for organic matter and so arsenic released in shallow, low permeability beds to have polluted the underlying aquifers to a depth of 10–20 m over a geologically reason-

able period of 3000–9000 years, but not to a greater depth. The introduction of pumping creates stronger vertical components of flow, and induces leakage of both organic matter and arsenic into the underlying aquifers. When wells are screened close to peat beds, the result is inevitably to accelerate these processes and a breakthrough phenomenon is to be anticipated (Cuthbert, 1999).

IMPLICATIONS

Arsenic pollution by oxidation of arsenical pyrite is a mechanism that is valid for oxic environments, typically surface waters. It may apply to the subsurface where high-permeability allows polluted surface water access to the subsurface, as in Zimapán, Mexico (Armienta et al., 1997). It may apply where oxic conditions invade a previously anoxic environment, for example, one hosting sulfide ore such as occurs in northeastern Wisconsin (Schreiber et al., 2000), where a commercially prospective sulfide ore-body, up to 3 m thick, (A. Weissbach personal communication, 2000) is exposed to oxic conditions in domestic boreholes. Oxidation of the ore results in pollution of groundwater by high concentrations of arsenic (15,000 $\mu g\ l^{-1}$), sulfate (<618 mg l^{-1}), iron (<160 mg l^{-1}) and acidity (pH 2.1) (Schreiber et al., 2000; A. Weissbach personal communication, 2000). Despite the presence of this massive sulfide ore, wells cased through the ore zone, and drawing water from a few meters below it, contain less than 50 $\mu g\ l^{-1}$ of arsenic confirming that it is access of oxygen to boreholes that causes pyrite oxidation and arsenic pollution (A. Weissbach personal communication, 2000).

Where arsenic pollution occurs in most subsurface, and most anoxic, environments, the pyrite oxidation model is inappropriate and a different model is needed. Reduction of FeOOH (invoked before for groundwater, e.g., Gulens et al., 1979; Matisoff et al., 1982; Cullen and Reimer, 1989; Korte, 1991; Bhattacharya et al., 1997; Nickson et al., 1998, 2000; McArthur et al., 2001; refs. therein) will serve in most instances. As the process is generic and not site specific it should be tested for wherever naturally occurring arsenic pollution occurs in groundwater in alluvial aquifers, such as in Argentina (Nicolli et al., 1989; Garcia et al., 2000), Taiwan (Chen et al., 1994), China (Wang and Huang, 1994; Sun et al., 2000), Hungary, and the USA (Welch et al., 2000). In this context, it is noteworthy that the arsenic pollution of deep wells in Hungary is accompanied by high concentrations of ammonium, iron and methane (M. Csanady, personal communication, 2000), presumably derived from deeply buried peat deposits that drive iron reduction and arsenic pollution. Severe arsenic pollution has now been reported by Berg et al. (2001) in groundwater wells supplying the city of Hanoi, in the Red River Basin of Vietnam, wells analyzed in a recent survey of pollution (BGS, 1996) that did not include analyses for arsenic, a WHO-listed parameter for drinking water. The area around Hanoi hosts abundant peat deposits (BGS, 1996) and there are striking similarities in the high concentrations of bicarbonate, iron, phosphorus and ammonium in the groundwater in the vicinity of Hanoi and in arsenic-affected areas of Bangladesh. Arsenic pollution in Taiwan occurs in an environment that is interpretable as swampy and organic-rich, from the statement of Tseng et al. (1968, p. 460) that "Waters tapped from organic black sediments of the lagoons in the endemic area are full of soluble salts such as arsenic and methane gas".

It is likely that any fluvial or deltaic basin that has hosted marshland and swamp will be prone to severe arsenic pollution of borehole water. The specific Holocene environmental history of the Bengal Basin accounts for the particular geometry of arsenic contaminated aquifers in Bangladesh. However, a set of globally applicable parameters — the depth of pre-Holocene incision, the relative rates of sea level rise and alluvial aggradation, and the Holocene temperature and rainfall records — provide a general model for predicting the possible occurrence of arsenic in any basin. While concentrations of arsenic of, say, 2–30 $\mu g\ l^{-1}$ may prove quite common in recent alluvial aquifers in humid

tropical environments, frequent high concentrations (>200 $\mu g\ l^{-1}$) may require the special condition of extreme accumulation of organic matter. This condition may involve a flooded Holocene marine (or possibly lacustrine) platform to provide the accommodation space for paludal deposits, that in turn create the redox driver to mobilize large amounts of arsenic. In many areas of the world, agriculture and urbanization occur on lowland coastal plains in a setting similar in type, although not always in scale, to that in Bangladesh. Such areas might be expected to suffer from arsenic pollution, and it should be looked for. Vulnerable regions include the deltas of tropical rivers, such as the Mekong, Red, Irrawaddy, and Chao Phraya rivers, and the northern coastal plains of Java and Sumatra.

CONCLUSIONS

Neither pyrite oxidation, nor competitive exchange of fertilizer-phosphate for sorbed arsenic, cause arsenic pollution of groundwater in the Bengal Basin. Indeed, pyrite in Bangladesh aquifers is a sink for, not a source of, arsenic. Pollution by arsenic occurs because FeOOH is microbially reduced and releases its sorbed load of arsenic to groundwater. The reduction is driven mostly by the microbial metabolism of buried peat deposits. The subordinate role of human organic waste in latrines as a redox driver for arsenic pollution requires investigation. Dissolved phosphorus comes from FeOOH, as it is reductively dissolved, and from the degradation of buried peat deposits and subordinately, organic human waste in latrines. Dissolved ammonium in the aquifer derives mostly from the microbial fermentation of buried peat deposits and, subordinately, from degradation of organic human waste in latrines. Ammonium ion is not, therefore, a reliable indicator of fecal contamination. Reduction of FeOOH, and release of sorbed arsenic, serves as a generic model for arsenic pollution of aquifers where waters are anoxic because organic matter is abundant, e.g. in deltaic or fluvial areas that hosted peat basins during climatic optimums and/or periods of rapidly rising sea level.

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Progresses on Researches of Endemic Arsenism in China: Population at Risk, Intervention Actions, and Related Scientific Issues

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ABSTRACT

Since the discovery of arsenic poisoning caused by drinking water was found in Xinjiang Province on the continent of China in the 1980s, arsenism has become one of the most serious endemic diseases in China in the last two decades. Up to now, more than 20,000 people have been diagnosed with illnesses caused by drinking water or burning coal rich in arsenic. This paper aims to describe some new progress on the research and the intervention actions in China. The topics covered include arsenic exposure and population at risk, health promotion, arsenic mitigation and safe water programmes, molecular mechanism of carcinogen, children's susceptibility, medical usage of arsenic, and other related scientific issues.

Keywords: arsenic; endemic arsenism; exposure; population; intervention; reactive oxygen species (ROS)

INTRODUCTION

Worldwide concern with arsenic and its influence on human health has increased markedly in recent decades. Epidemic areas of arsenism resulting from environmental and occupational exposure have been reported from several Asian countries including Bangladesh, India, Thailand, Japan, Philippines, and China (Chen et al., 1999). The serious situations, exposure sources and characteristics of the chronic endemic arsenism in China were described at the Third International Conference on Arsenic Exposure and Health Effect held in San Diego, 1998 (Sun et al., 1999). This paper aims to introduce some new progress on the research and the intervention actions in China in the last two years.

Three main themes are covered: (1) *arsenic exposure and population at risk*; (2) *intervention actions*, including promotion of health education to prevent or reduce the exposure to arsenic, provision of safe drinking water for residents in endemic areas, and some useful technologies for arsenic removal; and (3) *scientific issues* that are closely related to defining the adverse effects of arsenic exposure.

ARSENIC EXPOSURE AND POPULATION AT RISK

Several seriously affected areas in China have already been reported in Xinjiang, Inner Mongolia, Guizhou and Shanxi Province since the 1980s. Up to now, over 20,000 cases have been identified as patients of arsenism. Further investigations suggested that an even greater quantity of population than expected is in danger of arsenic exposure from drinking water. According to the latest data provided by the Academy of Preventive Medicine of China (Table 1), nearly 5.63 million people in 29 out of 32 provinces of China still drink water containing high levels of arsenic, exceeding 0.05 mg/l, which is generally accepted as the maximum by the majority of countries all over the world, including the USA. As to the WHO guideline value of 0.01 mg/l (World Health Organization, 1993), the exposed population amounts to 14.66 million, of which 9.03 million or so are exposed to arsenic levels ranging from 0.01 mg/l to 0.05 mg/l.

A large number of epidemiological investigations have confirmed that high concentrations of arsenic are associated with skin lesions, peripheral vascular diseases, polyneuropathy, hepatopathy, diabetes mellitus, hypertension and a high risk of various skin cancers and internal cancers (Maloney, 1996). Moreover, an appropriate standard for arsenic in drinking water is still under discussion. Whether 0.01 mg/l should replace 0.05 mg/l as the standard has drawn much attention and caused many controversies among the researchers as well as the governments. North et al. (1997) suggested that the endemic areas can be divided into three groups: (1) high dose regions where people ingest from one to several milligrams of arsenic per day (above 0.5 mg/l) and where various skin and internal cancers and other health problems have been found; (2) medium or intermediate dose regions, where intake ranges from 100 μm to 1 mg per day (0.05–0.5 mg/l or so), where health

TABLE 1

Population exposed to arsenic from drinking water in China

Arsenic concentration (mg/l)	Population
>0.01	14,657,000
0.01–0.05	9,027,000
>0.05	5,630,000
0.05–0.1	3,342,000
>0.1	2,288,000

From Chinese Academy of Preventive Medicine.

TABLE 2

Arsenic concentrations in tube-well water in one village in Inner Mongolia

Arsenic levels (mg/l)	Numbers of wells	Percentage
≤0.05	162	53.5
≤0.01	77	25.4
0.01–0.05	85	28.1
≤0.05	141	46.5
0.05–0.1	27	8.9
0.1–0.5	104	34.3
0.5–1.0	6	2.0
>1.0	4	1.3
Total	303	100

The concentrations of arsenic are determined by hydride generation atom adsorption spectrophotometry (AAS).

outcomes were less certain or not as yet proven and in which epidemiological studies are essential; and (3) low dose regions with an intake of below or around 100 μm per day, corresponding to the level of the present US drinking water standard (0.05 mg/l). In China, besides the population exposed to fairly high arsenic in endemic areas, there is an even larger population exposed to arsenic concentrations of 0.01–0.05 mg/l. Further research should be focused chiefly on this latter population in order to decide which concentration (0.01 or 0.05 mg/l) should be selected as the final standard for arsenic in drinking water. Research on this aspect is now being carried out in China with the following objectives: (1) to confirm the arsenic latent health effects, especially the risk of various skin and internal cancers in different regions with various arsenic levels; (2) to offer data for issuing a proposed standard for arsenic in drinking water; (3) to provide a basis for evaluating the costs of changing the standard from 0.05 mg/l to 0.01 mg/l.

In China, several investigations on arsenic levels of tube wells have already been finished in some epidemic areas. A survey conducted in one village of Inner Mongolia, China, showed that of the total of 303 wells, 162 wells (53.5%) contained arsenic concentrations less than 0.05 mg/l, 141 wells (46.5%) exceeded 0.05 mg/l, while 131 wells (43.3%) contained arsenic ranging from 0.05 to 0.5 mg/l. As far as the standard of 0.01 mg/l is concerned, 74.6% of the total wells exceeded this level (Table 2).

Another large-scale investigation in the Basin of Datong and Jinzhong, Shanxi, China during 1994–1995 showed that of all the randomly selected 2373 wells distributed in the 129 villages, there were 837 wells with arsenic beyond the standard of 0.05 mg/l (Table 3). Four villages in Shanyin county, one of the most seriously affected areas in Shanxi Province, have more than 35,000 people exposed to high arsenic levels via well water. The arsenic concentrations in the majority of wells there were beyond 0.5 mg/l and even as high as 4.44 mg/l, which was about 88 and 440 times higher than the standard of 0.05 mg/l and 0.01 mg/l, respectively.

Up to now, the water mitigation programme has not been carried out extensively. People in the vast endemic areas of China are still in the danger of high arsenic exposure. In our investigation, we also observed that besides adults, some children had already developed hyperkeratosis on their palms and soles. What is more, a large population has been exposed to arsenic over a period of 15–20 years in most of the endemic areas of China. They are at a very high risk of developing arsenic-related cancers. All these problems should be treated very seriously.

TABLE 3

Arsenic concentrations in tube-well water in 129 villages in Shanxi Province

Counties	Numbers of wells	Arsenic levels (mg/l)			Wells with arsenic beyond 0.05 mg/l	Percentage
		0.05	0.05–0.50	0.50		
Shanyin	2062	1348	593	122	715	34.6
Yingxian	74	55	19	0	19	25.7
Shuocheng	86	42	44	0	44	51.2
Tianzhen	28	15	13	0	13	46.4
Fenyang	64	29	35	0	35	54.7
Xiaoyi	27	20	5	0	5	14.8
Pingyao	32	30	2	0	2	6.3
Total	2373	1539	711	122	833	35.3

The arsenic levels are determined by silver diethyldithiocarbamate (SDDC) spectrophotometry at 510 nm.

INTERVENTION ACTIONS

The serious situations of arsenism in China have aroused much attention among many countries and international organizations including USA, Japan, WHO, UNICEF, etc. Collaborate research for the prevention and treatment of arsenism are being broadly carried out.

Health Promotion

Arsenism in China is mainly found in the less developed areas with poor living conditions and backward culture. The residents there may know that drinking water or burning coal with high arsenic concentrations will cause diseases. However, they do not have a clear understanding about it. In response to this challenge, our actions are mainly as follows: (1) to put up posters and send out pamphlets to spread widely the knowledge on the causes, exposure pathways, adverse health effects, prevention and treatment methods of endemic arsenism; (2) to make field tests of the arsenic concentrations in wells in endemic areas. Every well examined was determined as a 'safe well' (As $\leq$ 0.05 mg/l) or a 'dangerous well' (As $>$ 0.05 mg/l), then marked correspondingly by a green or red color as symbols. This is the easiest way for residents to consciously drink safe water. In the coal burning areas, however, the residents are being persuaded to cook and dry foods and peppers with chimneys. Therefore, the degree of arsenic exposure may be decreased remarkably. (The projects above are now being supported by UNICEF.)

Due to the poor living conditions in the endemic areas, the relatively short history of arsenism and the uncompleted water mitigation programme in China, such intervention actions mentioned above proved to be economic and effective. However, to take the necessary actions, one of the most imperative things now is a rapid and accurate field test of every well. We at present use a field test instrument called Arsenator (also provided by UNICEF) whose sensitivity is 0.01 mg/l and can determine the arsenic concentration in the water within 15 minutes. However, despite of its efficiency, the usage of the instrument is still limited by its high cost. To solve this problem, we are seeking help and collaboration from the international organizations and from other countries.

Arsenic Mitigation and Safe Water Programme

To provide safe drinking water is the fundamental and ultimate step for the sake of resident in the endemic areas. In some serious regions, the water mitigation programme funded by our government has been carried out successfully, so that residents there are now drinking safe tap water instead of tube-well water. They are no longer been

exposed to arsenic and the arsenic-related symptoms have been relieved to some extent. However, only for a limited range of endemic areas the water improving projects have been completed .

On the other hand, in the regions having no capacity of replacing tube-well water by clean and safe tap water, a small-size apparatus, the Reverse Osmosis System (OSA Kemflo), is now being used instead. This apparatus can provide 50–150 l of pure water per day which will serve 100–300 persons. We are under the discussion with UNICEF to place the apparatus in every primary school of endemic areas to protect children from arsenic exposure via drinking water.

Several well-known methods for removing arsenic from water have been successfully used by other countries with arsenic contamination problems. The method of activated alumina absorption in India is very effective since activated alumina can bind arsenic strongly, thus arsenic in the contaminated water can be removed completely when passing through the activated alumina filter. Further research on effective arsenic removal technologies like this still need to be developed in China.

SCIENTIFIC ISSUES

At present, arsenic pollution is one of the key public health problems of China. The major scientific researches in recent years are introduced as follows.

Carcinogen Mechanism of Arsenic

Inorganic arsenic is an established human carcinogen and is associated with various tumors including lung, skin, and other internal cancers in exposed populations. However, generally accepted animal models for arsenic-induced cancers have not been demonstrated. Many studies have shown that arsenic can affect DNA repair and methylation, increase the generation of reactive oxygen species (ROS), activate the protooncogen such as c-myc, and so on, but none of these potential pathways have been convincingly accepted as the principal etiology (Abernathy et al., 1999). The carcinogenic mechanism of arsenic remains unclear.

Some recent evidences have suggested that certain genotoxic effects of arsenic may be mediated by ROS (Lee and Ho, 1995). In the endemic areas of Inner Mongolia, we have found that 8-hydroxy-2′-deoxyguanosine (8-OH-dG), one of the widely accepted sensitive markers of oxidative DNA damage, was excreted in the urine of arsenic exposed people. This is in accordance with the study by Matsui et al. (1999) which demonstrated that 8-OH-dG was significantly higher in people with arsenic-related skin neoplasms and arsenic keratosis. Our further analysis showed that of all the subjects examined, there were positive correlations of urine 8-OH-dG levels with total arsenic (tAs) and dimethylarsenic (DMeAs) levels in whole blood, respectively. What is more, increased urine 8-OH-dG levels correlated positively with the ratios of (MMeAs + DMeAs)/iAs in whole blood and negatively with the ratios of iAs/(MMeAs + DMeAs) in urine (MmeAs is monomethylarsenic and iAs is inorganic arsenic). 8-OH-dG is generally considered to be one of the main oxidative base damages to DNA, and may cause mutation (G : C to T : A) during DNA replication process. Therefore, we are now going deeply into the involvement of ROS and the role of 8-OH-dG in arsenic-related malignancies. Further studies will be carried out to clarify the dose–effect relationship between arsenic ingestion and 8-OH-dG in urine, blood and peripheral leukocyte of exposed population, and to determine whether 8-OH-dG can serve as a sensitive marker of arsenic exposure and arsenic-related malignancies.

Importance of Arsenic Ingestion in the Burning-Coal-Type Endemic Areas

It is generally reported that chronic endemic arsenism in China is caused either by drinking well water with high arsenic content or by breathing arsenic-containing burning coal. However, new information (Li et al., 1999) suggests that in addition to burning coal, nearly half of the total amount of arsenic is ingested from peppers. Peppers are one of the main foods of the residents in the endemic areas and are inclined to accumulate arsenic. So when residents dry peppers with coal rich in arsenic, peppers are contaminated with arsenic. The amount of pepper ingested there is about 10–30 g per person per day, hence about 0.5–1.5 mg arsenic per person per day is from pepper ingestion. It is an important source of arsenic exposure in Guizhou Province, China. For the prevention of arsenism, it is extremely necessary to change the residents' habit of drying peppers by burning coal rich in arsenic.

The Combined Toxicity of Fluoride and Arsenic

In some chronic endemic areas of arsenism, fluoride is also abundant in well water. The combined poisoning of fluoride and arsenic is of an exceptional type in China which has received interest all over the world. Some studies suggested that a synergistic interaction existed between the two elements. However, there is no consensus on the mechanism of the combined toxicity up to the present.

Children's Susceptibility

Children sometimes differ from adults in their susceptibility to hazardous chemicals. In studies of chronic arsenic exposure, children appeared to be more severely affected, probably due to a higher exposure per body weight. The characteristic skin lesions from arsenic have already shown in children of some regions in China. Moreover, the cases of hyperkeratosis in children are increasing remarkably in some regions we have investigated recently. Children's susceptibility to arsenic should be treated seriously.

Medical Usage of Arsenic

Whether arsenic is a trace element essential to human health remains a subject of debate. However, many studies have proved the potential beneficial effects of arsenic (ATSDR, 1998). In traditional Chinese medicine, the arsenic compound Xionghuang(AsS) was recorded as one of the superior materials in 'Bencaojing', a famous classic work of pharmacology written during the Ming dynasty. It is reported that arsenous acid or arsenic trioxide (As_2O_3) is effective in cosmetology, longevity and is often used against many diseases such as psoriasis, syphilis, rheumatosis, and trypanosomiasis (Chen et al., 1995). It was in the 1970s in China that As_2O_3 was introduced into the treatment of acute promyelicytic leukemia (APL) and it showed a striking effectiveness. A clinical complete remission rate was reported (Sun et al., 1992; Zhang et al., 1996) from 65.6% to 84% with treatment (10 mg/day, intravenous infusion for 29 to 60 days). In vitro studies suggested that As_2O_3 may induce the apoptosis of the NB_4 cell by down-regulating the bcl-2 gene expression and altering the localization of PML-PARa/PML protein (Chen et al., 1996). In recent research in China it has also been observed that As_2O_3 can induce apoptosis in the cell lines of hepatocarcinoma. It is a medical breakthrough and may open a brand-new pathway in the treatment of malignancies.

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Arsenic in Latin America: Occurrence, Exposure, Health Effects and Remediation

Ana M. Sancha, María L. Castro

ABSTRACT

In order to show the Latin American experience in arsenic issues the authors review available regional information. The arsenic occurrence in surface and groundwater in some Latin American regions has been associated with the tertiary and quaternary volcanic processes of the Andes, sulfide ore deposits, geothermal activity, mining and smelting activities and use of pesticides. Studies in Chile and Argentina suggest that ingestion of inorganic arsenic from drinking water results in increased risks of internal cancers, particularly bladder and lung cancer. Latin American researchers have experience in arsenic removal from water supplies. Arsenic has been removed from drinking water for almost 30 years with simple and reasonably priced technologies and recently a low-cost and easy technology point-of-use device has been developed and tested under local conditions. The arsenic occurrence, exposure, health effects and remediation in different countries are discussed.

Keywords: arsenic; Latin America; hydroarsenicism; arsenic removal; arsenic exposure; health impacts

INTRODUCTION

The natural presence of As in Latin America's surface water and groundwater is associated with the tertiary and quaternary volcanism developed in the Andes, manifested in flows of lava, geysers, fumaroles, thermal waters, and geothermic phenomena related to the 'fire circle of the Pacific' (Henríquez, 1968, 1978). This geological condition generates important mining activities in the Andean region.

Arsenic is present in surface water and groundwater as a result of geological and anthropogenic factors derived from mining activities and to a lesser extent, from the deposition of organic pesticides containing arsenic (Table 1). Arsenic can be found in water both in its trivalent and pentavalent form, according to environmental conditions. In surface water, oxidized forms prevail and in groundwater, especially in less shallow ones, it is found in its reduced form. Methylated forms have not been reported. The water chemistry is characterized by high pH, variable alkalinity, low hardness, high to moderate salinity and the presence of boron, fluorine, silica, and vanadium.

SOURCES OF ARSENIC

Argentina

In Argentina, arsenic is of natural origin and is mainly found in groundwater at concentrations ranging from less than 0.10 mg/l to over 1 mg/l. The provinces of Cordoba, Chaco, Santiago del Estero, Salta, Buenos Aires and La Pampa have important areas with high contents of As (Trelles, 1920; Trelles et al., 1970; Callegaro et al., 1976; Schulz et al., 2000).

Bolivia

The main drinking water source of La Paz receives waste water from the mining area of Milluni. A study determined that in this zone soils and drinking water contain arsenic.

TABLE 1

Arsenic sources in Latin America

Country	Arsenic source	
	natural	anthropogenic
Argentina	X	X
Brazil	X	X
Bolivia	X	X
Chile	X	X
El Salvador	X	
Mexico	X	X
Peru	X	X
Brazil	?	
El Salvador	?	
Colombia	?	
Ecuador	?	
Venezuela	?	
Uruguay	?	
Paraguay	?	
Guatemala	?	
Honduras	?	
Nicaragua	?	
Costa Rica	?	
Panamá	?	

There is also information about presence of As in rivers, lakes and ponds located in the southwest of Bolivia (Quintanilla, 1992; ECO/PAHO, 1997).

Brazil

The main sources of arsenic are industrial wastes and atmospheric emissions of arsine generated in the electrolytic production of Zn and Cd. High arsenic concentrations have been found in sediments near the industrial dumps at Ensenada Enseño (Barcellos et al., 1992; ECO/PAHO, 1997).

Chile

Arsenic is present in the northern and central area either by natural or anthropogenic conditions derived mainly from copper mining. The highest levels of contamination occur in the region located between 17º30′ and 26º05′ southern latitude and between 67º00′ western longitude and the Pacific Ocean. In these regions most surface water and groundwaters and soils present high concentrations of arsenic. Scarce crops in the area also reflect the presence of this pollutant (Henríquez, 1968, 1978). Additionally the intense mining activity, mainly smelters, generate important atmospheric emissions of As (Wiertz and Rozas, 1996). Therefore, the population in the north of Chile lives in an arsenic-polluted environment, the Andean population being the most affected (Alonso, 1992; Sancha et al., 1995).

Mexico

Arsenic is present in groundwater of the Región Lagunera in the central part of the north of Mexico and in the city of San Luis Potosí located in the surroundings of a copper smelter (Cebrián et al., 1994).

Peru

In the semi-arid region of southern Peru, arsenic-polluted water is drawn from Andean rivers and is consumed by the population; the most representative is the Locumba River (0.5 mg As/l) that passes through Puno and Moquegua. The main domestic and industrial water source of Ilo is the Aricota Lake. Its tributaries, the Callazas and the Salado, pass through the Yucamane volcano, which is apparently the contamination source (Kirchmer and Castro, 1984)

El Salvador

Arsenic is present in the water of Ilopango Lake.

Other Countries

Of Colombia, Venezuela, Ecuador, Uruguay, Paraguay, Costa Rica, Panamá, Nicaragua, Guatemala and Honduras no information is available

ARSENIC EXPOSURE AND POPULATION AT RISK

The geographical conditions of the Andean area of Latin America, characterized by high altitude, lack of water resources and adverse climatic conditions has limited to a great extent the development of large urban populations. Therefore, except for Chile, Peru, Argentina and Mexico, with a total of 3,150,000 persons affected, the population exposed in other countries should not be significant (Table 2).

Argentina

It is estimated that the population exposed to water with arsenic in a range between 0.002–2.9 mg/l is 2,000,000 in the provinces of Salta, La Pampa, Cordoba, San Luis, Santa Fe, Buenos Aires, Santiago del Estero, Chaco and Tucuman (Pinedo and Zigarán, 1998). Total exposure has not been estimated.

TABLE 2

Population exposed to arsenic in Latin America

Country	Population exposed
Argentina	2,000,000
Bolivia	25,000
Chile	500,000
Mexico	400,000
Peru	250,000
Brazil	?
El Salvador	?
Colombia	?
Ecuador	?
Venezuela	?
Uruguay	?
Paraguay	?
Guatemala	?
Honduras	?
Nicaragua	?
Costa Rica	?
Panamá	?

Mexico

Water supplies with As concentrations > 0.05 mg/l have been identified in Baja California Sur, Región Lagunera (Chihuahua, Coahuila, Durango, Zacatecas), Guanajuato, Hidalgo, Morelos, Acambaro, Delicias, Jiménez, Chihuahua, and San Antonio. A total of 400,000 people is estimated to be exposed, but the exposure has not been studied (Cebrián et al., 1994; Armienta et al., 2000).

Chile

The two cities of the northern area with higher exposure are Antofagasta and Calama (Sancha et al., 1998). The population exposed to contamination by arsenic in these cities is about 500,000.

The experience of Chile is quite particular because it is similar to an experimental design during a specific period of time in a well-defined population of very low migration. For approximately 20 years, the population has been supplied with water containing arsenic concentrations in the range of 40–50 μg/l. Another 40 years are required to evaluate excess cancer risks associated with this exposure, since carcinogenic effects have a latent period. This information could be used to evaluate the validity of the 0.05 mg/l standard for the population.

Studies show that in the north of the country the most important source of arsenic exposure is drinking water, as well as in Santiago and Rancagua, located in the central area. In southern Chile, exposure through diet is of greater importance. As for air, in general it contributes very little to the total exposure (Sancha and Frenz, 2000)

Peru

It is estimated that the population exposed is of 250,000 people. There also exists a high exposure to arsenic in workers from the mineral deposit of La Oroya, but it has not been quantified.

Bolivia

The communities at risk are located towards the north of El Alto, in the Murillo province, department of La Paz, and in the community of Vinto, in Oruro. The total exposed

population is about 20,000. Studies have determined that the most important exposure routes are soil, water and dust and that children are the population at a greater risk (ECO/PAHO, 1997).

Brazil

Exposure to arsenic is present in Lamarao do Passe, at 4 km from a copper smelter (favorable wind conditions) and in the bay of Sepetiba, in the west coast of Rio de Janeiro (Machado et al., 1992; ECO/PAHO, 1997).

HEALTH EFFECTS: NON-CANCER AND CANCER

Since the late 1920s, Latin American researchers have been reporting health effects caused by polluted water consumption. Although many of these publications do not meet the current requirements for scientific publications, they have described the health effects observed in the last 80 years. These effects correspond to both acute and chronic effects (Table 3).

The health effects derived from exposure to As reported in the medical literature by Latin American researchers refer to adverse effects in skin, including hyperkeratosis of palm and sole, hyperpigmentation and skin cancer, peripheral vascular diseases consisting of myocardial infarctions and arterial enlargement in the case of children and recently cancer of internal organs. The prevalence of unspecified injuries to skin has been reported widely in that population. There is a clear exposure–response relation between As concentrations and frequency of skin injuries. Recently, Chile and Argentina showed epidemiological evidence of association between mortality due to cancer of internal organs and ingestion of water polluted with As (Rivara and Corey, 1995; Smith et al., 1998; Ferreccio et al., 1998; Hopenhayn-Rich et al., 1998).

Argentina

Since the early years of the last century studies related to chronic arsenic contamination have been published (Ayerza, 1918; Alvarez, 1928). Recent studies report that keratosis is more frequent than hyperpigmentation and that most patients have some form of skin cancer. Of the total of internal cancers diagnosed, 66% were lung cancer (ECO/PAHO, 1997). Illnesses reported include hyperkeratosis, melanosis, leukodermia, basal cell carcinoma, senile keratosis, and a high incidence of cancer of the bladder and urethra. Cases of arsenic

TABLE 3

Arsenic Exposure in Latin America: health effects

Country	Health effects	Reference
Argentina	Hyperkeratosis, melanosis, lenkoderma, basal cell carcinoma, senile keratosis and high incidence of cancer of the bladder and urethra.	Ayerza, 1918; Trelles, 1920; Hopenhayn-Rich et al., 1998; Pinedo and Zigarán, 1998
Bolivia	Skin lesions and neurological disorders.	ECO/PAHO, 1997
Chile	Leukedermia, melanosis, hyperpigmentation. Respiratory and cardiovascular diseases. Cancer (lung, kidney, bladder, liver and skin).	Borgoño and Greiber, 1971; Puga et al., 1973; Rosenberg, 1974; Zaldivar, 1974; Morán et al., 1977; Rivara and Corey, 1995; Ferreccio et al., 1998; Smith et al., 1998
Mexico	Skin lesions, hyperpigmentation, skin cancer.	Diaz-Barriga et al., 1992; Armienta et al., 1994; Cebrián et al., 1994

poisoning from water were reported in Tucuman, also in the province of Cordoba (Pinedo and Zigarán, 1998).

Mexico

In addition to the classical signs and symptoms of hydroarsenicism, some studies suggest that the prevalence of black foot attains 0.7% among the population at risk (Díaz-Barriga et al., 1992; ECO/PAHO, 1997).

Bolivia

The effects of child exposure to arsenic in El Alto area are skin injuries and neurological disorders. Another study in the Vinto mining region, 7 km from the city of Oruro, showed that children were the population at greatest risk of suffering neurological problems (ECO/PAHO, 1997).

Brazil

The existing information refers to exposure to smelters emission and shows the accumulation of arsenic in the hair of women in the town of Lamarao do Passe where the atmospheric arsenic levels were 5.1 ng/m^3 . Another study showed high levels of As in urine of foundry workers (ECO/PAHO, 1997).

Peru

A study involving workers of three smelters, two of them in Lima and one in the Andes (La Oroya) reported arsenic-related disorders. High average concentrations of arsenic in the air reached 2.42 and 110 mg As/m^3, respectively. The maximum acceptable limit at that time was 0.5 mg As/m^3.

Chile

The first reports pointed at background documentation of children with clear signs of arsenicism: leukodermia, melanodermia and hyperpigmentation. Respiratory and cardiovascular problems have also been described (Borgoño and Greiber, 1971; Puga et al., 1973; Zaldivar, 1974; Rosenberg, 1974; Morán et al., 1977).

In the eighties the first data on the major risk of lung cancer in Antofagasta were known. Background information showed a SMR of lung cancer of 503 for Antofagasta (5 times higher than the expected). These observations are currently valid, when new studies have excluded the potentially confounding factors, such as occupational exposure, migrations, tobacco, and other exposures (Rivara and Corey, 1995).

Recently the health effect of arsenic exposure has been evaluated by ecological studies, case studies and cross checks of cancer mortality rates associated with arsenic (lung, kidney, bladder, liver and skin). These have been carried out by both national and international researchers based on the complete medical and statistical records in the country (Smith et al., 1998; Ferreccio et al., 1998).

TREATMENT TECHNOLOGIES FOR ARSENIC REMOVAL

Technologies used in Latin America for arsenic removal from water supplies include chemical precipitation through coagulation filtration and softening. Adsorption in local materials (hematite, bone, clay) has also been tested.

Argentina

An arsenic removal method was developed thirty years ago (Trelles et al., 1970; Callegaro et al., 1976; Dodero et al., 1978). Recently chemical coagulation studies have been conducted at the water treatment plant of Pompeya in San Antonio de los Cobres, province

of Los Andes. Water with arsenic levels ranging from 0.27 to 0.30 mg/l was treated with aluminum sulfate, lime and polyelectrolyte. The arsenic concentration was reduced to 0.12–0.15 mg/l. However, the plant needs to be adapted to treat larger volumes (Figueroa and Montes, 1995).

In the province of Santa Fe, a pilot filter was implemented for water containing 0.27 mg As/l and using direct coagulation; the arsenic content of drinking water was reduced (Mozziconacci et al., 1998). The Alufloc technology developed by PAHO/CEPIS is also being used successfully for arsenic removal at the household level (Esparza, 1996).

Mexico

The following proposals and studies on arsenic removal from water have been developed.

(1) The Instituto Mexicano de Tecnología del Agua adopted a methodology based on the coagulation–flocculation process to remove arsenic from drinking water; the arsenic concentration in water was reduced to nearly 0.05 mg/l (Bedolla et al., 1999).

(2) In the state of Durango, PAHO recommended for rural and urban areas a technology using crushed bone, lime and aluminum sulfate. For urban areas, especially the central area of Mexico, the adoption of alternative sources and water treatment using crushed bone, activated aluminum, and reverse osmosis was recommended (Solsona, 1985, 1986).

(3) A pilot study on arsenic removal carried out in Zimapan, Hidalgo, identified hematite as an alternative sorbent for removal. The study was conducted in well V of Zimapan that supplies 47% of the water distributed to the population, and which has an arsenic content ranging from 0.5 to 0.9 mg/l. Field results confirmed the effectiveness of hematite in arsenic removal, resulting in concentrations of less than 0.05 mg/l in the water treated (Petkova, 1999).

Chile

Arsenic removal technologies were first applied in the 1970s to treat surface water sources in Antofagasta (Sancha and Ruíz, 1984). Currently, there are four As removal plants that treat 2000 l/s with 0.40 mg As/l and produce drinking water with 0.040 mg As/l (Karcher et al., 1999; Sancha and Frenz, 2000). Still, it is necessary to solve the problem for the scattered population, comprising mainly indigenous groups, that consume water with high concentrations of As (0.600 mg/l). Simple methods of As removal have been tested, but no solution has yet been adopted according to the needs and possibilities (Sancha et al., 1992).

Chile's arsenic removal experience at water treatment plants indicates that arsenic can be removed to residual concentrations of 0.030–0.040 mg/l through coagulation using $Al_2(SO_4)_3$ or Fe Cl_3, and that arsenic removal is more effective when using Fe Cl_3 as the coagulant. The key elements for successful As removal are pH and alkalinity of raw water, the chemical state of arsenic and separation processes of the floc in which As has been adsorbed.

Arsenic removal at household level indicates that certain problems should be recognized and solved, such as pre-conditioning of raw water, scarce water for filter washing, lack of electric power, climatic conditions, cultural aspects, basic sanitation conditions, lack of skilled personnel, the economic situation, etc. Arsenic removal is only part of the solution for the scattered population; water disinfection and health education programs should be the first priority (Sancha, 1999).

In the case of Antofagasta and Calama, cities located in the north of Chile, arsenic removal from drinking water sources was recently evaluated as well as its costs. The study made a technical and economic evaluation of the following options: improvement of water treatment, introduction of reverse osmosis as a post-treatment of the effluent from the existing process and installation of a desalinization plant whose effluent should be mixed

with the water produced by the existing treatment system (Sancha et al., 1998; O'Ryan and Sancha, 2000)

Peru

In the city of Ilo, the treatment plant built in 1982 was designed to remove arsenic and turbidity from water. Initially, the treatment involved the use of high doses of lime; but it was subsequently improved by the use of new treatment methods which reduced costs, enhanced efficiency and improved the quality of the water treated. The possibilities considered were iron chloride, iron hydroxide and sulfuric acid and arsenic removal with high pH, coagulation and flocculation with natural $Mg(OH)_2$ (Kirchmer and Castro, 1984).

CONCLUSIONS AND RECOMMENDATIONS

In Latin America there are approximately 3,150,000 people exposed to arsenic in drinking water, both in urban and rural areas. The populations that present the highest risk are the rural and sparsely distributed population, the aboriginal population and mine workers.

Arsenic is not a new pollutant in Latin America; our ancestors lived with it. We have been removing it from drinking water since the 1970s using simple and cost-effective technologies which may reduce the arsenic in drinking water to 0.040–0.050 mg/l. We are aware that more advanced technologies should be used to reach the level of 0.010 mg/l recommended by the World Health Organization. This will result in high treatment costs and will require careful operation. Is it possible to sustain this in Latin America?

Environmental issues like arsenic contamination should go beyond the theoretical level. Sometimes theory and practice prove to be different. New epidemiological studies and the development of a reliable and more accessible analytical capacity are necessary. In brief, basic research should be undertaken and existing results should be disseminated to avoid duplication of efforts and the optimization of scarce financial resources. Latin America has plenty of information and experience, share it!

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Impacts of Historic Arsenical Pesticide Use on Residential Soils in Denver, Colorado

David J. Folkes, Steven O. Helgen, Robert A. Litle

ABSTRACT

A variety of arsenical compounds were used as pesticides in the U.S. during the first half of the 20th century. Until recently, the impacts of these practices were thought to be limited to orchard soils and other agricultural lands. However, recent investigations of metal contamination of soils near a smelter have revealed that use of a widely available crabgrass killer and insecticide (PAX) during the 1950s and 1960s resulted in arsenic concentrations of up to several thousand parts per million in the soils of residential properties over a large area of Denver, Colorado. Discrimination of pesticide and smelter impacts was a challenging process. Impacts due to smelter emissions were finger-printed by concentrations that decreased with distance, maintained constant metal ratios, and were consistent with modeling of historic emissions and deposition. Pesticide impacts were identified by their random distribution, spatial patterns that were controlled by property line features, and arsenic concentrations that were disproportionately high compared to other metals emitted by the smelters. Air photo analysis showed that high arsenic concentrations associated with pesticide use only occurred on developed properties with well established turf. PAX was finger-printed by the presence of commercial-grade arsenic trioxide crystals, co-location of high arsenic and the perlite filler used in PAX, and arsenic to lead ratios similar to the product formula. Similar arsenic contamination likely exists on numerous residential properties throughout the Denver area and in other cities with similar climatic and soil conditions. These findings raise important public health, logistical, and funding questions.

Keywords: arsenic trioxide; PAX; lead arsenate

INTRODUCTION

Recent investigations near a former smelter site in Denver, Colorado, have revealed high arsenic concentrations in soils on certain residential properties due to the historic application of a commercial crabgrass killer during the 1950s and 1960s. Although the potential impacts of arsenical pesticides on soils have been known for many years (e.g., McMurtrey and Robinson, 1938), cases in the literature have been largely confined to orchards and not residential properties without a history of this land use. Further, many of the arsenic concentrations caused by herbicide use in Denver significantly exceed those due to smelter emissions and likely occur in other neighborhoods of the city. This paper discusses the nature of the herbicide contamination and the forensic methods that were used to separate impacts due to herbicide application from those due to smelter emissions.

The pesticide impacts were inadvertently discovered while investigating soil contamination around a former smelter in Denver, Colorado. The ASARCO Incorporated (Asarco) Globe Plant was originally built in a rural area in 1886. Today, the site is located within an urban area of the city along the South Platte River valley, a major transportation and industrial corridor with a mixture of residential, commercial, and industrial land use (Fig. 1). Soils in the site vicinity are neutral to alkaline sandy to clayey loams with organic concentrations typically exceeding 1% in the surface horizons and clay accumulations in the B-horizons (SCS, 1974). The climate is semiarid with a mean annual precipitation of 13.8 inches and a mean annual temperature of 52 degrees F (TRC, 1988).

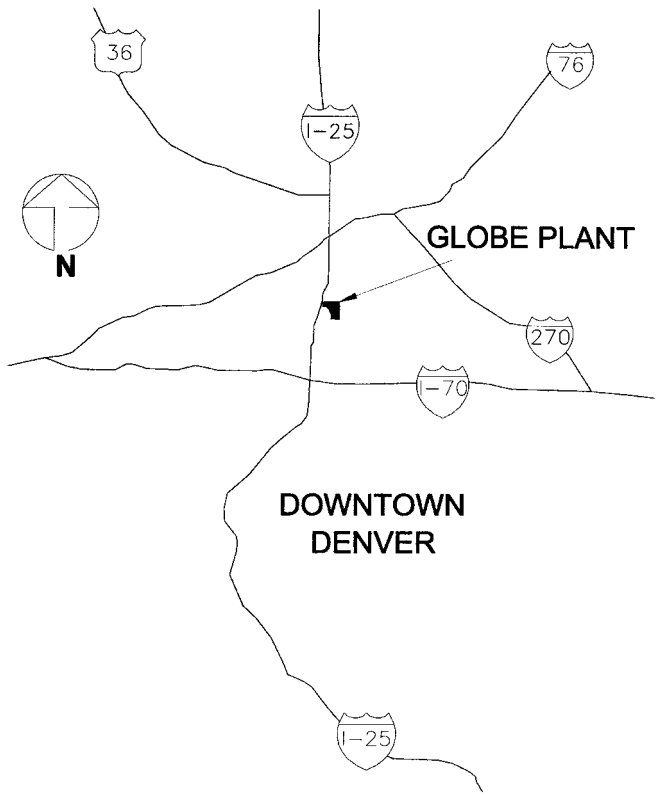


Fig. 1. Site location.

The Globe Plant smelted ore to produce lead bullion, silver, and gold until 1919, when lead-smelting operations ceased. From approximately 1912 until 1927, the plant produced arsenic trioxide by refining bag house dust from other lead smelters. From 1927 until 1993, cadmium refining was the principal operation at the Globe Plant. Since 1993, only relatively small scale, high-purity metal refining has been conducted at the plant.

The State of Colorado sued Asarco in 1983 under the Natural Resource Damages (NRD) provision of the Comprehensive Environmental Reclamation, Cleanup, and Liability Act of 1980 (CERCLA). In an effort to settle the lawsuit, a Remedial Investigation and Feasibility Study (RI/FS) was carried out between 1985 and 1990 to identify, among other things, the nature and extent of soil contamination in the surrounding community due to historic emissions of arsenic, cadmium, lead, and zinc from the facility (TRC 1988, 1990). Soil cleanup or action levels for each metal were set by the State of Colorado (CDH, 1993) based on risk assessment calculations (Putnam, 1989). The primary action level for arsenic is 70 mg/kg, while a voluntary or secondary arsenic action level was set equal to the 'upper limit of background' for arsenic, or 28 mg/kg. The primary action levels for cadmium and lead are 73 mg/kg and 500 mg/kg, respectively.

The community soil cleanup program was subsequently defined by a Statement of Work (SOW), as part of a Consent Decree entered by the U.S. District Court on July 15, 1993 that settled the NRD lawsuit. The SOW requires sampling of every property surrounding the Globe Plant in each direction until a 'clean block' is reached. A provision in the SOW allows Asarco to petition the State to not clean up properties that were contaminated by other sources not related to the Globe Plant.

Yard-by-yard soil sampling began in late 1993, as described below. Within two years, very high arsenic concentrations exceeding levels adjacent to the Globe Plant were found at distances beyond the expected area of smelter impact. The remaining sections of this paper describe the forensic process that eventually identified historic application of a crabgrass killer on lawns as the source of these anomalous arsenic concentrations.

INVESTIGATION PROCEDURES

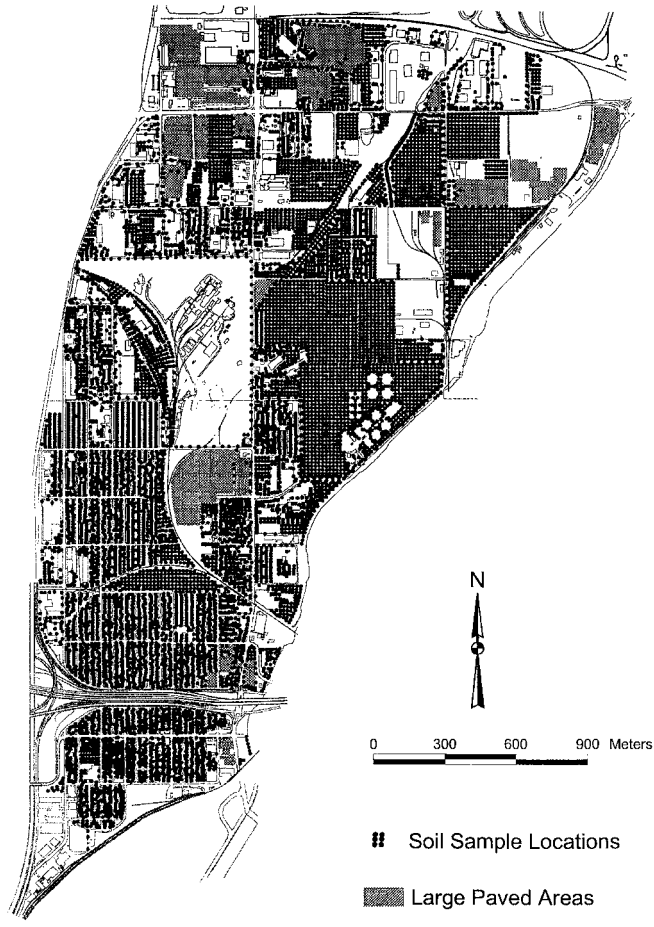
RIFS Investigation

RI/FS soil samples were collected at approximately 150 locations within two miles of the Globe Plant over 0 to 5 cm and 5 to 20 cm depth intervals, based on a polar grid system that resulted in a greater sample density near the plant. The sampling program was deliberately biased or stratified to select sites with the least amount of apparent disturbance (e.g., vacant lots) to maximize the likelihood that metals due to deposition of historic air emissions would still be present and not removed or obscured by recent development or landscaping activity (TRC, 1988). Therefore, established lawns and other landscaped areas were not sampled during the RIFS. Discrete samples were collected at each site from random locations within an 8 m by 8 m area judged to be relatively undisturbed. The minus 2 mm fractions of all soil samples were analyzed for arsenic, cadmium, lead, and zinc concentrations. Arsenic analyses were conducted by EPA Methods 206.1 and 206.2, with a detection limit of 0.5 mg/kg. More detailed information is provided in TRC (1988, 1990).

SOW Investigation

To date, over 20,000 samples have been collected at residential and commercial properties over an area of approximately 5 km^2 during the SOW cleanup program (Fig. 2). Properties were divided into at least four sectors not exceeding 2500 ft^2 (232 m^2) in size. Core samples were collected at four random locations within each sector over the 0 to 5 cm and 0 to 15 cm depth intervals and composited to create one sample for laboratory analysis for each depth interval in each sector. The samples were air-dried and sieved through a #10 mesh screen (minus 2 mm fraction) prior to aliquoting for digestion by SW-846

Fig. 2. SOW soil sample locations.



Method 3050B. Samples were then analyzed for arsenic, cadmium, lead and zinc by either inductively coupled plasma–atomic emission spectrometry (ICP) by EPA Method 6010A (6010B after 1998) or laboratory X-ray fluorescence (XRF), with arsenic detection limits of 6 and 10 mg/kg, respectively, following standard EPA method laboratory quality control procedures. During the time period the XRF was used, one in twenty samples were split and also analyzed by ICP, with ICP arsenic concentrations generally being about 5 to 10% lower than XRF concentrations. This difference in the two techniques should not affect any of the observations or conclusions presented herein, which are based on very large concentration differences between properties. The results of all tests are available in the Design Investigation Reports (DIRs) for the project submitted by EnviroGroup Limited every year to the Colorado Department of Public Health and Environment beginning in 1994.

Arsenic Anomaly Investigation

When the results of the SOW investigations revealed the presence of anomalously high arsenic concentrations on various residential properties south of the Globe Plant, additional investigations were conducted to determine the source of the anomalous arsenic.

TABLE 1

Chemical and physical test methods

Parameter	Test method
Arsenic, cadmium, lead, zinc, phosphorus	EPA 6010A/B, XRF
Total Kjeldahl nitrogen	EPA 4500
Ammonia	EPA 350.3
Nitrate	EPA 300.0
Organic matter	ASTM D 2974
Cation exchange capacity	EPA 9081
pH	AASHTO T289
Organochlorine pesticides	EPA 8081
Gradation – sieve and hydrometer	ASTM D 422-63
Atterberg limits	ASTM D 4318-95

Additional samples were collected at various depth intervals on selected anomalous and neighboring properties using core samplers and digging instruments, without compositing. Samples were analyzed for arsenic, cadmium, lead, and zinc by ICP, as described above, and for other chemical and physical parameters as shown in Table 1. Arsenic phases were determined by computer-controlled scanning electron microscope (CCSEM) analyses on one gram of minus 250 micron dry soil, performed in backscattered electron imaging mode using aluminum and carbon backscatter intensity standards, at 200× and 600× magnification. Petrographic analyses were performed using stereomicroscope to detect mineral phases in soil samples. Perlite was identified by analyzing samples prepared in 1.550 refractive index liquid by polarized light microscopy and comparing glassy fragments to known perlite standards, which are isotropic and have a refractive index lower than 1.550. More detailed information on these procedures is provided in EnviroGroup (1997).

RESULTS OF INVESTIGATIONS

The results of the RI/FS investigations indicated that arsenic concentrations decreased from levels of approximately 100 to 200 mg/kg near the Globe Plant to levels typically 20 mg/kg or less within 2000 m of the site (Fig. 3a). Cadmium concentrations decreased with distance in a similar manner (Fig. 3b). Similar findings were expected at the start of the SOW investigations. Indeed, cadmium concentrations during the SOW investigations did decrease with distance from the Globe Plant (Fig. 4a), consistent with the results of the RI/FS investigations. Arsenic concentrations, however, continued to remain elevated during the SOW investigations and even increased with distance, as shown in Fig. 4b. In fact, arsenic concentrations were much higher at properties more than 1000 m from the Globe Plant than found near the Globe Plant during either the RI/FS or SOW investigations, exceeding 1000 mg/kg on several properties (Fig. 5).

The impacts of smelter emissions on soil generally decrease rapidly with distance from the site because of dilution caused by the geometrically expanding air volume, plume dispersion due to air turbulence, and plume depletion due to fallout, as observed at numerous smelter sites reported in the literature (e.g., Balsley and Caldwell, 1974; Bolter et al., 1974; Temple et al., 1977; Ball et al., 1983; Kuo et al., 1983; Brams, 1985; Karczewska et al., 1998; and others). Because the randomly located, high arsenic concentrations encountered during the SOW were not consistent with air emissions from the Globe Plant smelter, further investigations were conducted to determine the source of the anomalous arsenic.

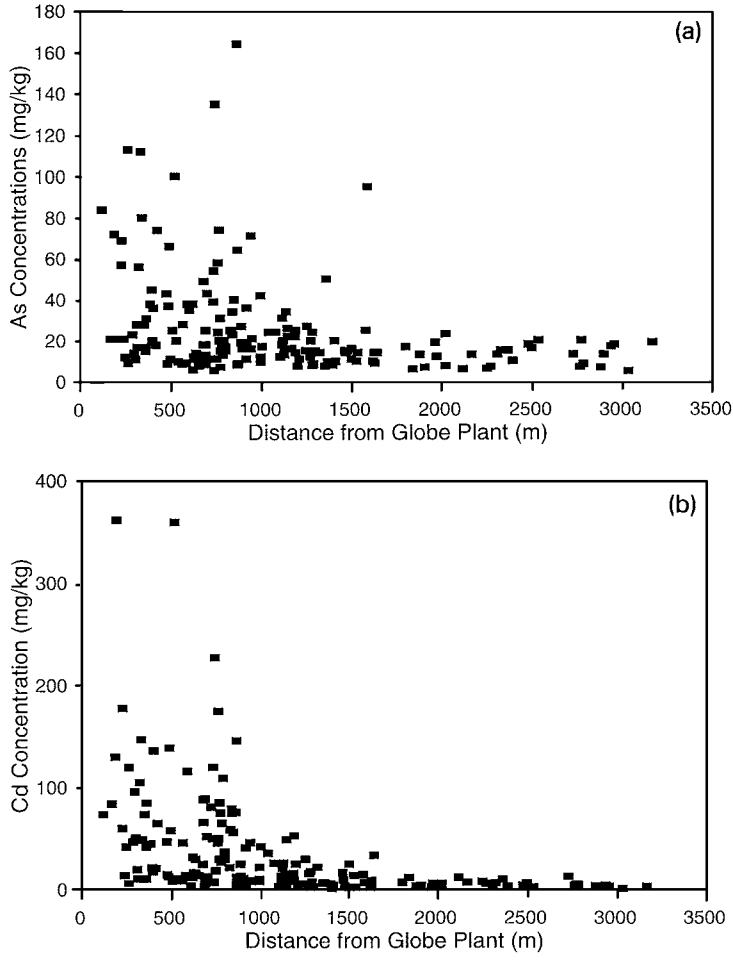


Fig. 3. (a) RIFS arsenic concentrations vs. distance. (b) RIFS cadmium concentrations vs. distance.

SOURCE EVALUATION

Potential sources of the anomalous arsenic concentrations that were considered during the source evaluation included air emissions from the Globe Plant (perhaps under gaseous transport conditions), application of pesticides, and placement of contaminated fill. A variety of evaluation procedures were used to fingerprint the source of the anomalous arsenic concentrations by testing for these various characteristics, as described below.

Spatial Patterns

Kriging analyses were conducted to see whether spatial correlations would indicate the source of the anomalous arsenic. On a large scale, cadmium concentrations decrease with distance, as discussed above, and show strong correlations with the Globe Plant and predominant northeast–southwest wind directions (Fig. 6a), consistent with a smelter emission source. Arsenic concentrations, however, are randomly distributed and, except in the immediate vicinity of the Globe Plant, have no spatial relationship with the plant

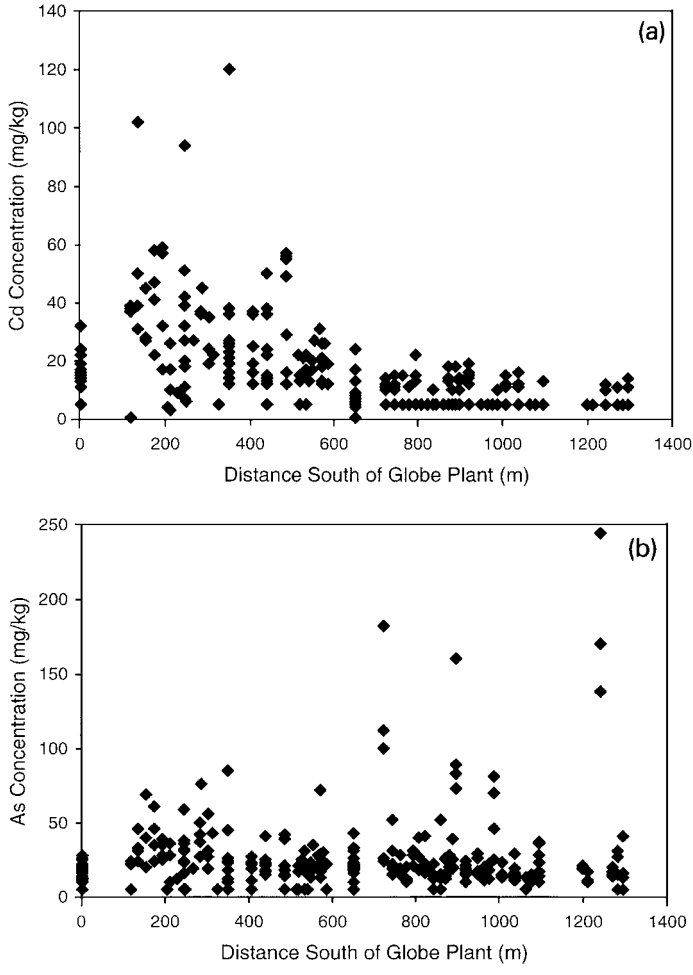


Fig. 4. (a) SOW cadmium concentrations vs. distance. (b) SOW arsenic concentrations vs. distance.

site (Fig. 6b). Most elevated arsenic concentrations are found in the southern, residential portion of the study area, while no anomalous arsenic concentrations are observed in the predominantly commercial and industrial area to the northeast (and downwind) of the Globe Plant.

The data set collected in the neighborhood contained several samples per yard allowing interpolation on a much finer scale than is generally performed in environmental investigations. A variogram analysis indicated an east–west anisotropy on a scale of 100 ft east–west, and 50 ft north–south, which corresponds with the approximate dimensions and orientation of individual residential properties. In order to incorporate the observed anisotropy into a kriging interpolation of the data, a search ellipse of 100 × 50 ft (30 × 15 m) was used with a 10 × 10 ft (3 × 3 m) grid. The resulting map of soil arsenic shows rectangular areas of arsenic that correspond with residential property boundaries (Fig. 7), strongly suggesting an arsenic source that was applied to specific properties and not to others. Furthermore, the east–west anisotropy observed in the data is inconsistent

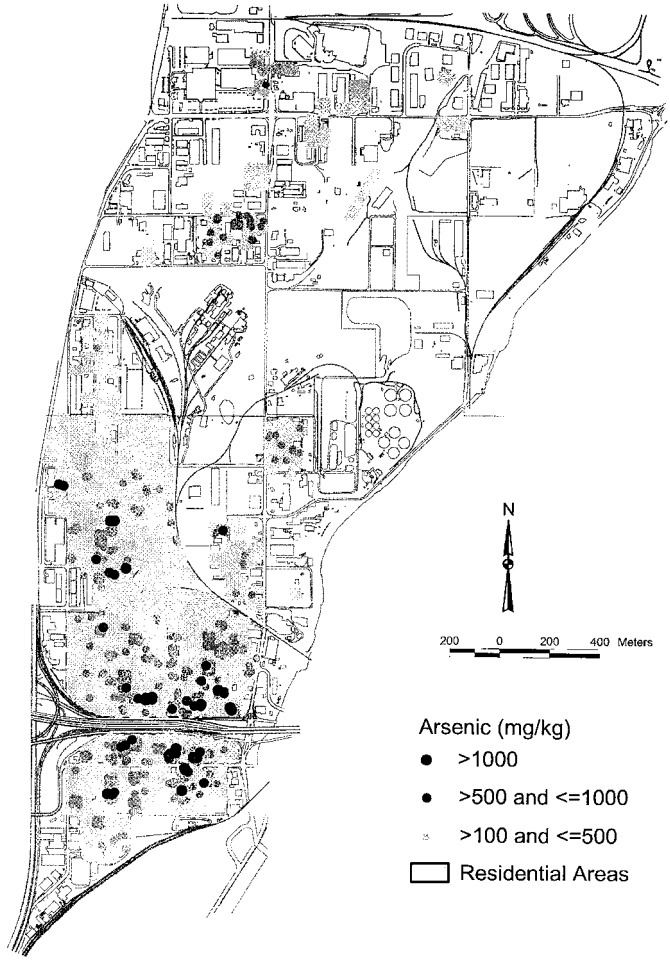


Fig. 5. Arsenic concentrations on residential properties.

with an airborne source located to the north, where the Globe Plant is located. The most striking feature of the map of soil arsenic is the readily apparent grid pattern of the arsenic corresponding with property boundaries, streets, alleys, and vacant lots.

These spatial patterns are consistent with pesticide application or contaminated fill placement on residential properties, and not airborne deposition, unless arsenic concentrations were altered after deposition by property-specific events, such as soil removal, fill placement, or activities that would enhance (or delay) the subsequent leaching of arsenic into the soil (see below). On the other hand, applications of arsenical products would be more likely to follow property boundaries.

Correlation with Land Use

Airborne emissions are expected to impact all types of properties, whether developed or undisturbed. This was observed to be the case for cadmium, when concentrations were compared to land use (Fig. 8a). A comparison of arsenic concentrations and land use,

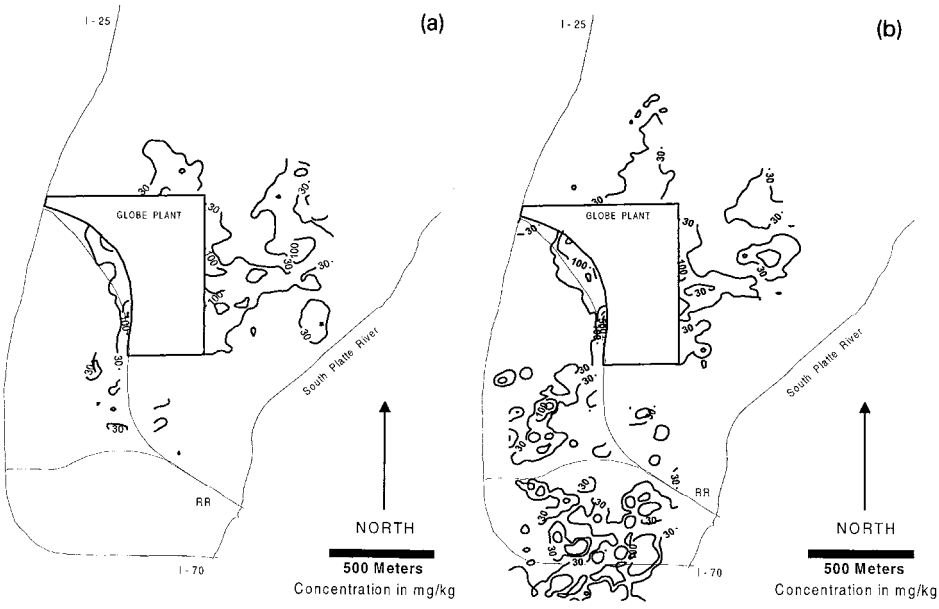


Fig. 6. (a) Kriging contours of cadmium concentrations. (b) Kriging contours of arsenic concentrations.

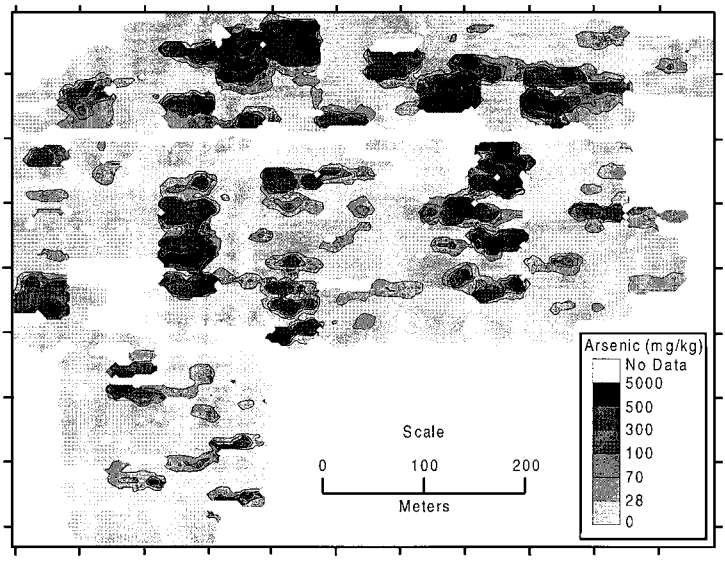


Fig. 7. Small-scale arsenic kriging contours.

however, indicated that anomalous arsenic concentrations only occurred on developed residential properties. Arsenic concentrations on undeveloped land (e.g., historically vacant lots, parks) and historically commercial properties (excluding properties where Globe Plant wastes and materials were directly placed by mechanical or waterborne means)

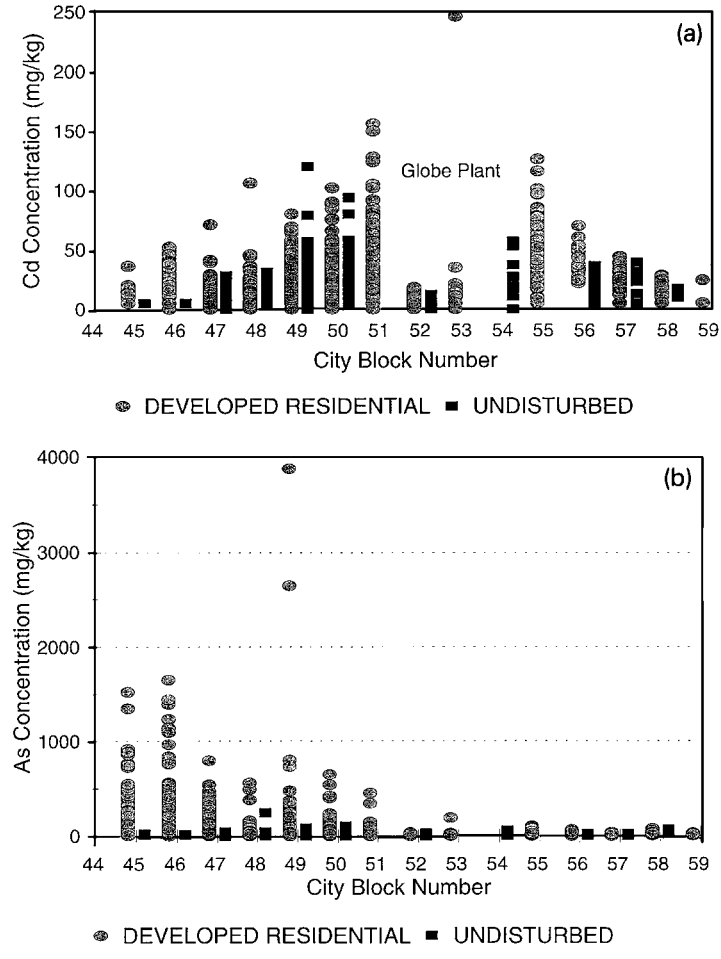


Fig. 8. (a) Cadmium concentrations vs. land use. (b) Arsenic concentrations vs. land use.

were low (Fig. 8b). This bias toward developed properties explains why the RIFS samples, which were collected from relatively undisturbed sites, did not include anomalous arsenic concentrations (Fig. 3a). The correlation between the anomalous arsenic concentrations and developed residential properties is not consistent airborne deposition but is consistent with the application of herbicides to residential lawns or the placement of contaminated fill.

The correlation between anomalous arsenic concentrations and land use was examined on a smaller scale by looking at two blocks where both developed residential and historically vacant lots were present. Prior to plotting the arsenic data (to avoid biased interpretations), historic aerial photographs were used to delineate the portions of properties on these two blocks where well-established turf was present in the 1950s and 1960s. Arsenic concentrations that exceeded 70 mg/kg (the action level) were then plotted. As shown in Fig. 9, all arsenic concentrations exceeding 70 mg/kg fell on sectors with well-established turf, consistent with the application of herbicides on lawns and inconsistent with airborne deposition.

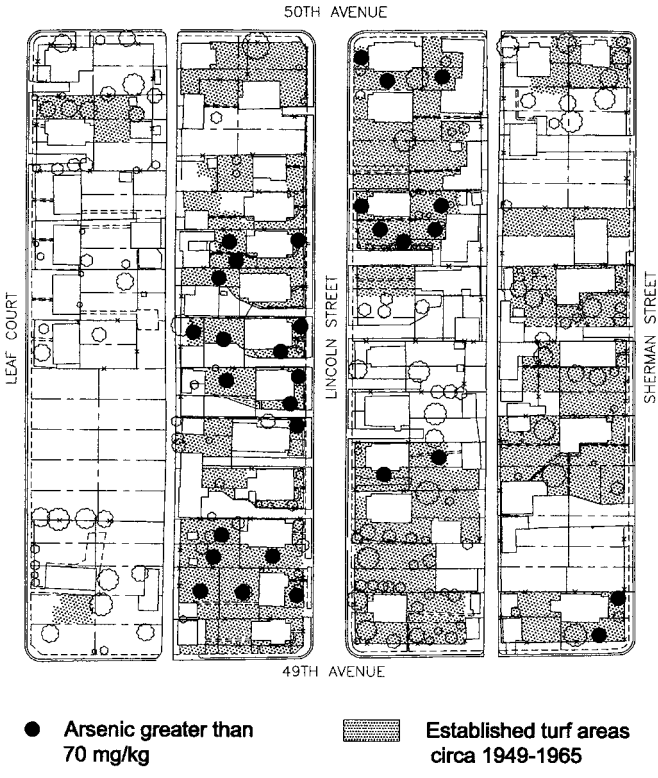


Fig. 9. Arsenic correlation with well established turf.

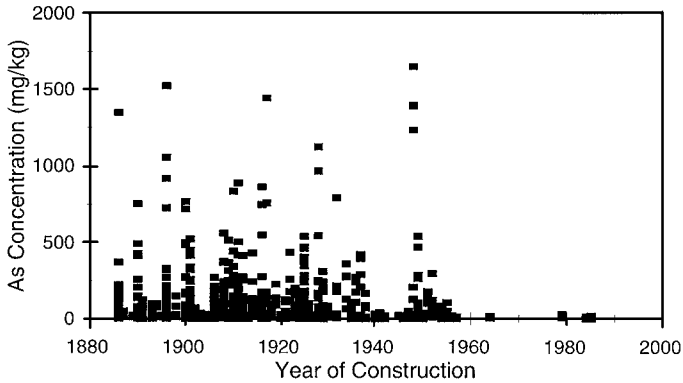
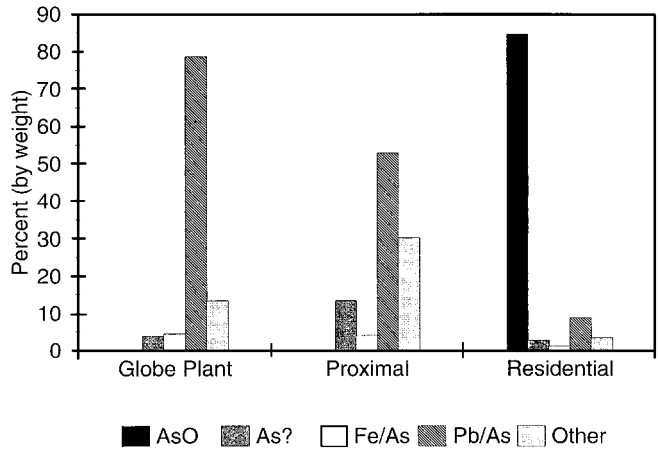


Fig. 10. Arsenic correlation with construction date.

Correlation with Construction Date

Arsenic concentrations were compared to the date of initial property development, based on tax records. Anomalous arsenic concentrations were observed on properties developed from the 1880s through the early 1950s (Fig. 10), with no particular trend over time. Because the highest arsenic concentrations were found in the 0–5 cm depth interval,

Fig. 11. Arsenic phases.



including the root mat of the lawns, it is safe to conclude that contamination occurred no earlier than the date of property development. The impacts of earlier contamination, while potentially existing at depth, would likely be disturbed by house construction and should not present in subsequently placed sod (see discussion of depth profiles, below). Therefore, at least some properties were being impacted as late as the early 1950s. If the arsenic had been applied at the time of house construction, we would expect the arsenic on older properties to be lower on average than arsenic on younger properties, because arsenic dissolution and leaching over time. Similarly, if the arsenic was the result of deposition over a long period of time, we would expect arsenic concentrations to be higher on older properties.

Because arsenic concentrations do not show any trend with the date of house construction, it is likely that all of the anomalous arsenic applications occurred after the early 1950s. This time period is not consistent with Globe Plant arsenic refining operations, which ended in 1927. While cadmium calcining operations included arsenic emissions, this time period is also not consistent with these operations because the greatest potential for emissions was in the 1930s and 1940s prior to the installation of modern air pollution control equipment. However, application of arsenical pesticides on lawns would have occurred in the late 1950s and 1960s (see below), consistent with the observed time trends.

Arsenic Speciation

Arsenic from smelter emissions and commercial pesticides might be expected to have different arsenic phases, or phase ratios. Therefore, soil samples were selected from residential properties with anomalous arsenic concentrations, the Globe Plant, and properties immediately downwind of the Globe Plant and analyzed by CCSEM. The arsenic on the anomalous residential properties was found to be predominantly arsenic trioxide, with small amounts of metal–arsenic phases, such as lead arsenate and clay mineral phases (Fig. 11). In contrast, no significant amounts of arsenic trioxide were found in Globe Plant soils or in the soils of properties immediately downwind of the plant (and likely to have been impacted by airborne emissions from the plant).

Arsenic trioxide was produced at the Globe until 1927. Arsenic was also emitted from the cadmium calcine stack during later years and white crystals (likely amorous form) were observed to form at the top of the stack and fall on the plant and nearby properties during the 1930s and early 1940s. Nevertheless, it appears that most residues of this process have dissolved over time and become associated with other metal and mineral

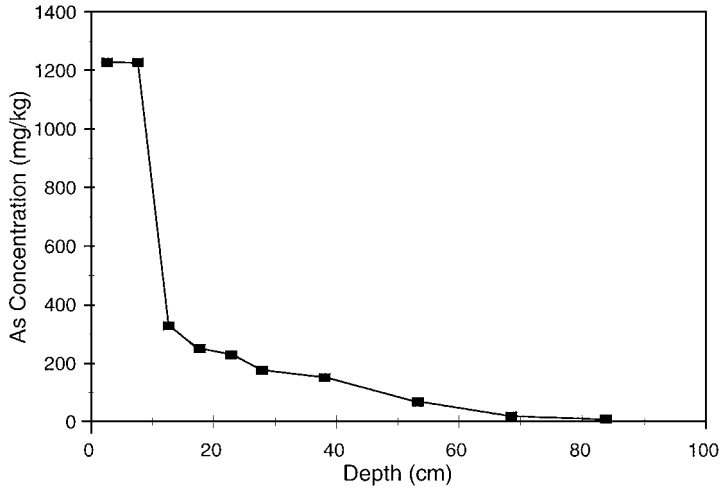


Fig. 12. Typical arsenic concentration depth profile.

phases. The arsenic trioxide observed in the anomalous soil was in the form of relatively large (up to and occasionally exceeding 100 μm) crystals (rather than amorphous) and more indicative of commercial product than emission particles.

Depth Profiles

Arsenic concentrations were measured at discrete depth intervals on several properties. In all cases where anomalously high arsenic concentrations were found in the upper 0–5 cm depth interval, concentrations decreased rapidly with depth (Fig. 12). This depth profile indicates that the anomalous arsenic concentrations resulted from surface application of arsenic, rather than placement of arsenic-contaminated fill, because concentrations in fill materials would tend to be random with depth throughout the fill zone. Evaporative forces (which can draw salts to the ground surface in arid climates) would not explain this depth profile, because the arsenic in the upper 0–5 cm was predominantly in the original arsenic trioxide crystal form (see discussion of arsenic speciation) and had not been dissolved. Further, the examination of neighboring properties with anomalous and low arsenic concentrations did not reveal the presence of any fill horizons that would explain the difference in arsenic concentrations.

While spatial and land use correlations indicate that the anomalous arsenic was applied to the surface individual residential lawns, additional data were required to evaluate the alternative hypothesis that arsenic due to airborne deposition was preferentially retained on (rather than applied to) these yards because of some property-specific condition. If this were the case, elevated arsenic concentrations would be expected at depth on properties where arsenic concentrations in the 0–5 cm depth range were low (i.e., by this hypothesis, already leached). Testing to depths of approximately 1 m did not reveal any significant arsenic at depth on several properties with low surficial values, including properties immediately adjacent to those with anomalously high arsenic concentrations, indicating that high arsenic concentrations never existed on these properties and that the preferential leaching (or retention) hypothesis is not valid.

Geochemical Profiles

If preferential leaching or retention of arsenic was the explanation for the property-specific distribution of the anomalous arsenic concentrations, some geochemical or textural char-

TABLE 2

Soil conditions at neighboring properties

Parameter	Property 44K05	Property 44K06
Maximum arsenic concentration (mg/kg)	12	1440
Dominant arsenic phases in upper 5 cm	Pb, Si–Fe	AsO (>90%), Pb
Soil type (Unified Classification System)	CL	CL
Plasticity index (%)	18	14–17
Organic matter (%)	3.9–6.4	3.3–4.2
Mineralogy	Similar	Similar
Perlite present	No	Yes
Chlordane, total (μg/kg)	97	198

acteristic should explain this difference in the leaching behavior. Therefore, metals concentrations, nutrients, pH, organic content, grain size, and Atterberg limits were measured at anomalous and neighboring, otherwise similar, residential properties with low surficial arsenic concentrations. As shown in the example in Table 2, the physical and geochemical properties of the soils were nearly identical. Further, no correlation was found between phosphorous, iron, or nitrogen and arsenic concentrations. Therefore, none of the data supported the hypothesis of historic, widespread arsenic contamination (e.g., by airborne emissions) that was subsequently leached at all properties but those with anomalously high concentrations today.

Correlation with Pesticide

Because of the evidence suggesting the anomalous arsenic had been applied to specific residential properties in the 1950s or later, as discussed above, the literature was reviewed for information on arsenical products applied to turf. Historians at PHR Environmental Consultants identified a lawncare product called PAX, which contained arsenic trioxide (25.11%) and lead arsenate (8.25%), and was used for crabgrass control in Denver and other cities in the 1950s and 1960s (U.S. Patent 3057709, 1962; Stadtherr, 1963; Frost et al., 1973). PAX was a solid, granular product that was typically applied by a drop spreader at rates of about 10 to 12 kg/100 m^2 of lawn, depending on the version of the product.

A single application at a rate of 12 kg/100 m^2 would theoretically increase the arsenic concentration in the upper 5 cm of soil by approximately 350 mg/kg (assuming a typical loamy soil dry density of about 1400 kg/m^3), while ten applications over a decade would theoretically increase arsenic concentrations by 3500 mg/kg. Dissolution and leaching of arsenic, however, would reduce concentrations in the upper 0–5 cm over time. Measured concentration profiles with depth (e.g., Fig. 12) indicate that approximately 30% of the arsenic was retained in the upper 5 cm at the time of testing, with the remainder typically distributed over the upper 30 to 60 cm (EnviroGroup, 1997). Therefore, modern-day arsenic concentrations of 100 to 1000 mg/kg are consistent with one to ten applications of PAX in the 1950s and 1960s, assuming that about 30% of the arsenic remained in the upper 0–5 cm. This range is consistent with the average observed range of anomalous arsenic concentrations on most properties. The occasional higher, individual concentrations are explained by overlap of drop spreader paths and deliberate over-application in areas with thick crabgrass. We can also imagine that some home owners applied PAX at rates that were higher than recommended due to miscalculation, incorrect spreader settings, a desire (perhaps misguided) for better performance, or to completely use up a bag of PAX (19 kg).

The arsenic to lead ratio in PAX would theoretically result in the following relationship between the total lead (Pb) and total arsenic (As) concentration in soil:

$$\text{Total Pb} = \text{Background Pb} + (\text{Total} - \text{Background As})/4.2 \quad (1)$$

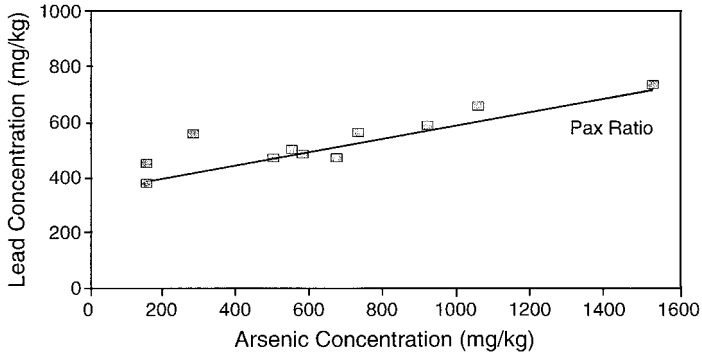


Fig. 13. Correlation with PAX lead/arsenic ratio.

The background lead concentration is the lead concentration prior to the application of PAX, due to natural soil levels and anthropogenic contributions (e.g., leaded gas, lead paint). If the background lead concentration was the same at all points on the property, the lead and arsenic concentrations after the application of PAX would fall along a straight line defined by equation 1. In reality, the background lead concentration will vary from point to point across a yard, resulting in departure from a straight line. Nevertheless, the lead and arsenic ratios in samples at several properties with anomalous arsenic concentrations were similar to the slope of this line, or the PAX lead to arsenic ratio, as shown in Fig. 13.

On other properties, we observed a greater scatter of the data, but always to the left of a bounding line described by the PAX lead to arsenic ratio. This behavior is consistent with increased leaching of the product, and preferential leaching of the more soluble arsenic trioxide component which would cause data points to shift to the left of the PAX line in Fig. 13. Therefore, the lead to arsenic ratios on the anomalous properties are consistent with historic applications of the PAX product, and are inconsistent with applications of pure arsenic trioxide or other metal-phase arsenic (which would tend to plot below the PAX line, or randomly throughout the plot space, respectively).

A strong correlation was found between anomalous arsenic samples and the presence of perlite, one of the inactive ingredients of PAX (U.S. Patent 3057709, 1962). All residential lawn soil samples with anomalous arsenic concentrations that were examined by stereomicroscope were found to contain perlite, while no perlite was found in any of the samples with low arsenic concentrations. While perlite is also found in other products, such as potting soil, the chances of this correlation occurring by random chance are negligible.

Testing in other Areas

If the anomalous arsenic concentrations found in Globeville are due to historic applications of PAX, it is reasonable to expect anomalously high arsenic concentrations in other parts of Denver where the product was likely used, i.e., older neighborhoods that would have lawns sufficiently aged to have crabgrass infestation in the 1950s or 1960s. While random testing of other neighborhoods was difficult for Asarco to justify, a property was discovered in south Denver, approximately 8 miles south of the Globe Plant, where a former resident recalled their family using PAX on the lawn. Fortunately, the property was still in the possession of the family and sampling access was granted. Elevated arsenic concentrations (maximum 210 mg/kg), lead/arsenic ratios that fit the PAX ratio, the presence of arsenic trioxide crystals, a decreasing arsenic concentration profile with depth, and the presence of perlite, all matched the PAX finger-print that was observed in Globeville soils.

Subsequently, the U.S. EPA listed the Vasquez Boulevard – I70 Superfund Site on the National Priorities List, which included several square miles of neighborhoods to the east of Globeville where similar high arsenic concentrations were detected. While investigations were not complete at the time of this paper, the data indicate that high arsenic concentrations occur on random properties across the neighborhoods, without any apparent spatial relationship to the Globe Plant or any other point source (ISSI, 2000). Intensive sampling of selected properties shows that the arsenic concentrations are confined to property boundaries, as observed in Globeville (ISSI, 2000).

CONCLUSIONS

Several lines of evidence indicate that anomalous arsenic concentrations found in the Globeville area are due to the historic application of the crabgrass killer, PAX, although application of other arsenical products, such as lead arsenate, likely contributed to the high levels on some properties. Based on testing in other neighborhoods, and because PAX was sold throughout Denver, it is reasonable to conclude that these impacts may be relatively widespread in the older neighborhoods. Further, similar impacts may be observed in other cities where PAX was sold, depending on climate and soil conditions (i.e., the degree of leaching).

To a large degree, Globe Plant smelter impacts can be separated from pesticide impacts by the spatial correlation of the former to the Globe Plant, and the lack of spatial patterns among the latter sites. In areas of overlap, examination of the metal trends on non-residential properties should provide a good indication of smelter impacts, while the presence of perlite might be used as an indicator of PAX on pesticide-impacted properties. Careful modeling of the dispersion and deposition of historic air emissions from the Globe Plant has also shown promise as a means of separating smelter and other urban sources of arsenic and other metals (EnviroGroup, 1998).

The presence of the pesticide impacts raises important socioeconomic questions. How prevalent are the pesticide impacts, and what is a reasonable approach to evaluating this condition on the thousands of properties that could potentially be impacted? If present, to what extent is the arsenic a source of exposure to people living on impacted properties, and to what extent is remediation, if any, necessary? Finally, what cost-effective methods are available for dealing with any identified concerns, and who will provide the funding?

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Arsenic Speciation in Fresh-Water Fish and Bivalves

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ABSTRACT

The major arsenic species in extracts of fresh-water fish from Yellowknife, Canada, are arsenobetaine, dimethylarsinate, arsenosugars, and some unknowns. The concentrations of the species as determined by using HPLC–ICP–MS depended on the extraction method, methanol/water or protease digestion. Arsenosugars dominate the speciation in fresh-water mussels. Arsenobetaine is the major extractable arsenical in fresh and canned salmon but most of the arsenic is not extracted by methanol/water.

Keywords: fresh-water; fish; bivalves; arsenic; speciation; arsenosugars

INTRODUCTION

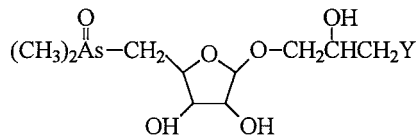
Although quite a lot is known about the arsenic species present in marine animals, there is a paucity of such information from the terrestrial and fresh-water environment (Cullen and Reimer, 1989). Some early work (Lawrence et al., 1986) suggested that the total arsenic concentrations in fresh-water fish were lower than those found in marine specimens. For example, fresh-water values range from 0.007 μg/g fresh weight (fw) (yellow perch), to 0.24 μg/g (fw) (striped perch). Typical marine fish concentrations range from 0.1 to 166 μg/g (fw) (Francesconi and Edmonds, 1997). Lawrence et al. (1986) were interested in the concentration of arsenobetaine in their samples and devised methodology to achieve this objective. They found arsenobetaine in their marine samples, as expected, accounting for 74–89% of the total arsenic. Salmon, which spend part of their life cycle in fresh-water was an exception in that arsenobetaine accounted for 0.15 μg/g (fw), 48% of the total arsenic content of 0.31 μg/g (fw), and an unknown arsenical, assumed to be organic, accounted for another 43% of the total. In contrast the fresh-water fish were found to contain no arsenobetaine but another unknown arsenical accounted for 71–84% of the total arsenic.

Arsenosugars such as 1a–1d, Fig. 1, were once believed to be largely confined to marine algae, although they were isolated from the kidney of the giant clam (Edmonds and Francesconi, 1993). However, in recent years, probably as a result of improved analytical methods, arsenosugars have been found in marine bivalves and fish (Edmonds and Francesconi, 1993; Francesconi and Edmonds, 1997), in terrestrial algae (Lai et al., 1997), terrestrial worms (Geizinger et al., 1998) and terrestrial plants and lichens (Kuehnelt et al., 2000). Thus it is to be expected that the anomalous absence of arsenobetaine from the fresh-water environment might also be the result of analytical oversight, especially since this arsenical is found in mushrooms (Byrne et al., 1995; Kuehnelt et al., 1997a,b; Slejkovec et al., 1997; Larsen et al., 1998; Koch et al., 2000), and plants and lichens (Kuehnelt et al., 2000). We now report that both arsenobetaine and arsenosugars are found in fresh-water fish and fresh-water bivalves.

EXPERIMENTAL

Fresh-water Fish

Fresh-water fish (whitefish, *Coregonus clupeaformis*; sucker, *Catostomus commersoni*; walleye, *Stizostedion vitreum*; and pike, *Esox lucius*) were caught from Back Bay, Great Slave Lake, near Yellowknife, NWT, Canada, using a gillnet, and they were gutted before freezing. The fish were thawed at a later date and filleted and skinned to obtain muscle samples, which were refrozen. All samples were stored in Ziploc® bags and freeze-dried before



	Y
1a	-OH
1b	$-OPO_3HCH_2CH(OH)CH_2OH$
1c	$-SO_3H$
1d	$-OSO_3H$

Fig. 1. Arsenic species found in the fresh-water environment.

extraction or digestion. Fish, fish roe, and oyster tissue certified reference material (CRM) 1566, obtained from NIST, were digested with protease, based on methods for enzymatic digestion (Forsyth and Cleroux, 1991; Branch et al., 1994). An accurately weighed 1 g (±0.5 mg) sample was combined with 0.02–0.05 g of protease (Type VIII, No. P-5380, Sigma) in a plastic 50-ml centrifuge tube. Ammonium carbonate (BDH) buffer at a concentration of 0.1 M and pH 7.2 (adjusted with nitric acid) was added and the tube was sealed and vortexed. The samples were shaken for 4 h at 37°C, then centrifuged, and the supernatant was diluted to 25 ml with deionized water. To determine the extent of arsenic solubilization by the protease, oyster tissue samples were extracted with buffer alone (no protease) and using the same procedure as that described for protease digestions.

For total arsenic determinations, fish and bivalve samples were acid digested and analyzed as described by Koch et al. (1999); methanol/water extractions and HPLC–ICPMS speciation analysis was carried out as described in this same publication.

Mussels

Mussels, *Margaritifera* sp., were collected from two sites near Campbell River, B.C., Canada. One site was a small stream south of the town and the other, the Quinsum River, north of the town. *Anadonta* sp. was collected from the Yellowknife River, near Yellowknife, NWT, Canada. The Campbell River samples were divided into two sets of five animals, and dissected. The muscle, foot, mantle, and remaining organs of each group were pooled and freeze dried prior to extraction with water/methanol (Lai et al., 1997). For total arsenic determinations, samples 0.050–0.200 g were weighed into 16 mm test tubes and digested in 2 ml of concentrated nitric acid in a heating bloc. The temperature was slowly increased from 70 to 150°C until the sample was dry. The residue was taken up in 3.00 ml of an aqueous solution of 1% nitric acid and 5 μg/l rhodium. Samples were mixed, filtered (0.45 μm), and stored frozen in Eppendorf vials prior to analysis.

The Yellowknife mussels were acid digested, protease digested, MeOH/water extracted, and analyzed as described above for fresh-water fish.

Salmon

A fresh wild sockeye salmon weighing approximately 2.5 kg and two cans of sockeye salmon were purchased from a local Vancouver market. The brine was decanted from the remaining contents and processed separately. Half the salmon was baked. The bones and skin were removed. All samples were freeze-dried and stored in a freezer until analysis.

RESULTS AND DISCUSSION

The Yellowknife environment is rich in arsenic because of high background levels (NRCC, 1978). Anthropogenic input has also been considerable because of extensive gold mining activity. The effluent from one mine, Royal Oak Giant Mine, flows down Baker Creek in the summer season into Back Bay, which is where the fish were caught.

The fresh weight values for the arsenic concentrations in Table 1 range from 0.025 μg/g (fw), for the pike to 0.132 μg/g (fw), for the sucker and are within the range reported by Lawrence et al. (1986). Another report (Jackson et al., 1996) of arsenic concentrations in fish from sites in Back Bay found an average of 0.385 ± 0.185 μg/g (fw), in pike muscle in agreement with the present results. The same is true for the data for whitefish. Jackson et al. (1996) also found that the arsenic content of whitefish, pike, and walleye muscle was dependent on the site, with high sediment values being associated with high arsenic levels: the highest sediment arsenic concentration was 1856 μg/g at the mouth of Baker Creek. Azcue and Dixon (1994) report that the average arsenic concentration in the white sucker from Moira Lake Ontario, Canada is 0.132 ± 0.047 μg/g (fw) and this bottom feeder has been suggested (Munkittrick and Dixon, 1989) as a sentential species for environmental

TABLE 1

Total arsenic concentrations in fish

Fish	Sex	Weight (g)	Moisture content (%) (R) [a]	Arsenic concentration (μg/g dry weight) (SD) [b]
Whitefish 1	Male	846	77 (4.33)	3.1
Whitefish 2	Female	586	76 (4.24)	0.84
Whitefish 3	Female	1453	74 (3.88)	0.28 (0.02)
Sucker 1	Male	656	78 (4.65)	1.24 (0.01)
Sucker 2	Male	457	81 (5.35)	0.98
Walleye 1	Male	386	79 (4.75)	0.46
Walleye 2	Male	507	77 (4.37)	0.85
Pike 1	Male	628	79 (4.72)	1.30
Pike 2	Male	988	80 (5.08)	1.40 (0.09)

[a] Moisture content was calculated by using $(R-1)/R \times 100\%$; R = fresh weight/dry weight.
[b] SD = standard deviation, based on duplicate acid digestions.

stress. The mean arsenic value for sucker from the Lower Columbia River is in the same range at 0.148 μg/g (fw) (Tera Tech, 1995).

In order to properly identify the arsenic compounds in biological samples it is desirable to release the arsenicals from the matrix into solution without changing their chemical form. This is often difficult to achieve, and certainly cannot be accomplished by the commonly used US EPA Method (U.S. EPA, 1996) involving digestion with HCl or NaOH. Such a method at best may give some indication of the content of inorganic arsenic in a sample; however, arsenosugars in particular would not survive such treatment (Edmonds and Francesconi, 1993). Extractions with, for example, methanol/water (1 : 1), is a method favored by us and others (Shibata and Morita, 1992); however, extraction efficiencies, EE (arsenic in extract/total arsenic), are variable. Thus, for example, the EEs of some dry algal products range from 5.8% to 98.3% (Lai et al., 1998) and the EEs of other marine samples can also be unsatisfactory (Shibata and Morita, 1992; Lai et al., 1999). With this in mind we wished to examine the use of a protease digestion to release the arsenic species from the fresh-water fish samples. Enzymatic digestion had been previously used by Branch et al. (1994) who used trypsin, a protease, to disrupt the lipid–protein membrane in fish samples. They found that, relative to methanol/chloroform extraction, recoveries were good for a certified reference material DORM-1 (dogfish muscle) and whiting, but recoveries were lower for mackerel and lemon sole.

The results in Table 2 show that the protease digestion is effective in releasing the

TABLE 2

Comparison of arsenic concentrations (SD) [a] by using protease and acid digestion methods for oyster tissue (NIST 1566) (The certified concentration of arsenic is 14.0±1.2 μg/g, and the non-certified value for Sb is 0.01 μg/g)

Digestion procedure	Concentration of As (μg/g)	Concentration of Sb (μg/g)
Acid digestion	14.0 (0.2)	0.3 (0.2) [b]
Protease digestion, pH 7.2	13.8 (0.2)	0.22 (0.08)
Buffer extraction, pH 7.2	11.4 (0.3)	0.17 (0.07)

[a] SD = standard deviation, obtained from 3 replicate extractions.
[b] SD for this sample was obtained from 2 replicate digestions.

TABLE 3

Concentrations of arsenic species in fish (μg/g, dry weight) (SD) [a]

Fish	Method [b]	As(V)	MMA	DMA	AB	Sugar 1b	Unknown	Sum of As species	% EE/DE [c]
Whitefish 1	PD	<0.01	trace	<0.01	0.28 (0.03)	<0.01	0.4 X [d]	0.69	22
Whitefish 2	PD	<0.01	<0.01	0.04 (0.03)	0.28 (0.13)	<0.01	0.11 X	0.43	52
Whitefish 3	PD	<0.01	<0.01	<0.01	0.052 (0.008)	<0.01	<0.01	0.052	19
Sucker 1	PD	0.05 (0.02)	0.03	0.023 (0.005)	0.25 (0.06)	0.15 (0.03)	0.19 Y [e]	0.70	56
Sucker 1	M/W	trace	<0.01	0.09 (0.06)	0.3 (0.1)	0.13 (0.03)	0.19 Y	0.72	60
Sucker 2	PD	<0.01	<0.01	0.029 (0.004)	0.24 (0.15)	0.11 (0.04)	0.19 Y	0.57	58
Sucker 2	M/W	0.03	<0.01	0.19 (0.06)	0.26 (0.06)	trace	0.26 Y	0.75	77
Walleye 1	PD	<0.01	<0.01	trace	0.04 (0.02)	<0.01	0.04 X	0.09	20
Walleye 2	PD	<0.01	0.03	trace	0.18 (0.06)	<0.01	0.05 X	0.27	32
Pike 1	PD	<0.01	<0.01	0.8 (0.1)	0.17 (0.08)	<0.01	0.6 Y	1.57	120
Pike 1	M/W	<0.01	<0.01	0.33 (0.02)	0.19 (0.07)	<0.01	0.23 Y	0.75	58
Pike 2	PD	<0.01	<0.01	0.5 (0.1)	0.22 (0.13)	<0.01	0.17 Y	0.89	64
Pike 2	M/W	<0.01	<0.01	0.38 (0.04)	0.26 (0.07)	<0.01	<0.01	0.64	46

Note: 'Trace' amounts are greater than or at the limit of detection (LOD) but less than 3 × LOD. Sum of As species includes trace amounts (given a value of LOD).

[a] SD = standard deviation, based on analyses of samples on 2 or 3 different chromatographic systems; see text for more detail.

[b] Method of sample preparation: PD = protease digestion; M/W = MeOH/water (1 : 1) extraction.

[c] % EE/DE = % extraction/digestion efficiency, calculated as (Sum of As species)/(Total arsenic from Table 4) × 100%.

[d] X = unknown arsenic-containing compound that corresponds to an early-eluting peak on the cation-exchange chromatographic system.

[e] Y = unknown arsenic-containing compounds that correspond to late-eluting peaks on the ion-pairing chromatographic system.

arsenic species from the oyster CRM so the method was deemed to be satisfactory for the fish samples. The protease digests of the methanol/water extracts were examined by using HPLC–ICP–MS. The results are shown in Table 3. Identification was made by comparison of the retention times with those of standards (Koch et al., 1999; Lai et al., 1999).

One notable feature of these results is the presence of arsenobetaine although it does not dominate the speciation as it invariably does in marine fish samples. Thus the failure of Lawrence et al. (1986) to find arsenobetaine is surprising. The major arsenical in the carnivorous pike is dimethylarsinic acid (DMA). This particular specimen had invertebrates and small fish in its stomach when caught. The presence of the arsenosugar 1b, Table 1, in the sucker is unusual (its presence in bivalves is described below). Arsenosugars have been found in only one other fish, a marine fish (silver drummer) that feeds on algae (Edmonds et al., 1997). Most of the arsenic in the silver drummer was as trimethylarsine oxide (rather than arsenobetaine) with arsenosugars 1b and 1d in the muscle, as well as the four arsenosugars 1a–1d in the digestive tract. The source of the arsenosugars in the silver drummer fish was postulated to be its food, brown and red algae. Suckers are bottom feeders and may also acquire arsenosugar 1b through their food, which implies that some benthic organisms are able to synthesize arsenosugars.

Two types of unknown compounds are present in these samples but we do not know if these correspond to those described by Lawrence et al. (1986). Unknown X in Table 3 was observed in very low levels in whitefish and walleye, and had an early retention time on the cation exchange system. It co-eluted with arsenate and arsenosugar 1b, but attempts to identify the unknown by using other chromatographic systems were unsuccessful. Unknown Y compounds (in Table 3) appeared as two late eluting peaks on the ion-pairing chromatographic system. These compounds did not co-chromatograph with

known arsenosugars found in kelp extract (Lai et al., 1999). Unknown compounds eluting at similar retention times (with respect to DMA) on the same chromatographic system have been observed as arsenosugar metabolites in human urine (Le et al., 1994).

Branch et al. (1994) observed that the trypsin digest of a single specimen of plaice contained more DMA than the organic extract and postulated that this could be the result of some degradation of arsenobetaine by the enzyme; however, no notable differences in arsenobetaine content in the sucker are seen between the protease digest and the methanol/water extracts, the same is true for the sugar and even the unknowns. The EEs are low and variable even with the same extraction method (note the results for whitefish 3). We expected the enzyme digest to be more efficient in releasing the arsenicals. However, there is the possibility that the released arsenicals are not detected in our analytical system. Such a situation is found even for methanol/water extracts (Lai et al., 1999).

Mussels

Data from the Mussel Watch Project (Valette-Silver et al., 1999) show that the national average for total arsenic in mussels and oysters is 11.1 ± 0 3.4 μg/g. A site is considered high in arsenic if the level in the bivalves is greater than 14.5 μg/g (one standard deviation above the national population geometric mean). The mean value for *Mytilus edulis* soft tissue from Australia is 3.0 μg/g (fw) and from Japan, 2.4 μg/g (fw) (Edmonds and Francesconi, 1993). Information about arsenic speciation from the Muscle Watch Project is meager, and what there is was obtained by using the US EPA method (U.S. EPA, 1996) so much is lost. Nonetheless the 'inorganic arsenic' in the samples ranges from the normal 1% of the total arsenic, to 20% in contaminated environments (Valette-Silver et al., 1999). Shibata and Morita (1992) have reported more complete data on arsenic speciation in marine mussels. *Mytilus edulis* contains arsenobetaine, DMA, and sugars 1a and 1b, Table 1. All of the organs of *Mytilus caruscum* contain arsenobetaine with most, 2.57 μg/g (fw), in the adductor muscle and least, 0.81 μg/g (fw), in the foot. Arsenosugar 1b is found in all compartments with most, 0.18 μg/g (fw), in the digestive gland and least, 0.03 μg/g (fw), in the mantle. The tetramethylarsonium ion is found in some compartments and sugar 1a only in the digestive gland: unknowns were also found in all compartments with most, 0.18 μg/g (fw), in the digestive gland.

Whole mussels, *Margaritifera* sp., collected from the Quinsum River (Campbell River, B.C.) had a total arsenic concentration of 3.1 μg/g. The mussel from Yellowknife was a different species, *Anadonta* sp., and the total arsenic content was 6.7 μg/g, so there may not be such a big difference between the arsenic concentrations in marine and fresh-water bivalves as is found for fish. These higher values suggest that these species might be used as indicators of arsenic exposure but much more information on organ distribution and arsenic speciation is needed to support such an undertaking.

In order to probe further the distribution of arsenic species in the mussels, determinations were made on selected body parts. The results are as follows for the Quinsum River samples (total arsenic, μg/g): muscle, 2.1; gills, 11.8; foot, 4.0; mantle, 7.0; remainder, 6.3. Mussels from the other Campbell River site showed a similar pattern for organ concentrations, but the overall numbers were lower. Speciation results for the methanol/water extracts of the Quinsum River samples are shown in Fig. 2. The EEs are low for all compartments. and range from 12% to 56%. The identification of the species is based on retention times and comparisons with known standards. The predominance of the arsenosugars should be noted, particularly the presence of the sulfate 1d. The uncertainty in the identification of the other arsenosugars is a consequence of matrix effects and low concentrations leading to poor chromatographic resolution. In view of the results for marine bivalves (Shibata and Morita, 1992) the species is most likely to be 1b. The presence of arsenobetaine is moot, which should be contrasted with the high concentrations of this arsenical in marine mussels as described above. A protease digest of whole mussels from

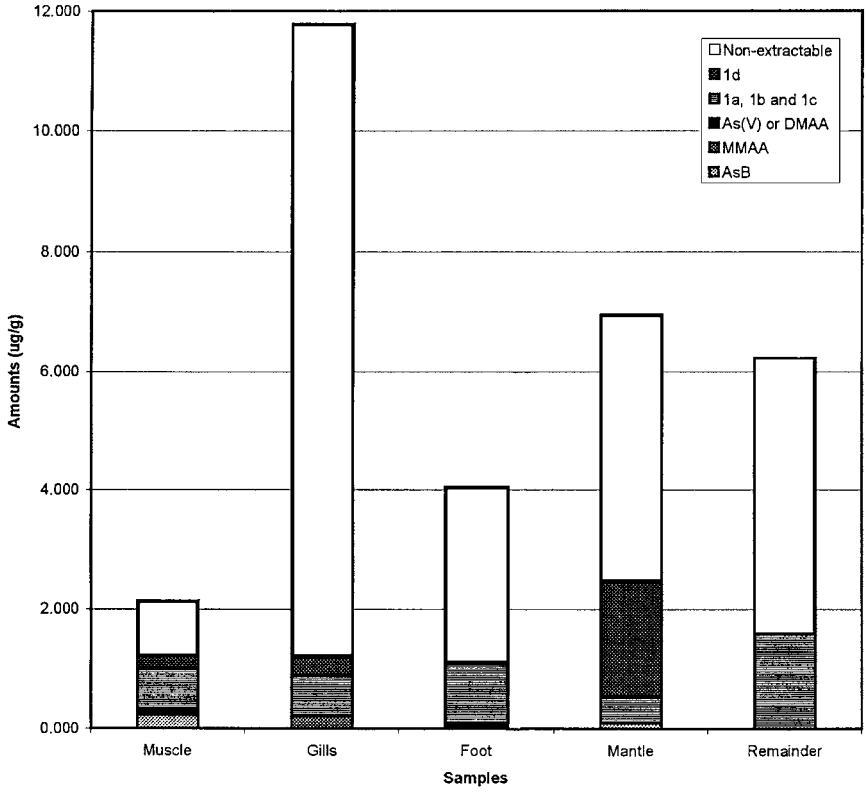


Fig. 2. Arsenic species (see Fig. 1) and their concentrations in organs of the fresh-water mussel *Margaritifera* sp.

the same site showed DMA 0.03 μg/g, 1a 0.05 μg/g, 1b 0.15 μg/g, as the major species. This particular sample did not contain 1d, which was absent also from the methanol/water extract. This extract also contained higher amounts of sugar 1a, 0.4 μg/g, and an unknown 0.11 μg/g. Again the EE was low at 42%.

The main species in the protease digest of the mussels from Yellowknife (August collection) were As(V) 0.13 μg/g, 1a 1.1 μg/g, 1b 0.3 μg/g. A methanol/water extract showed similar results except that more DMA was present together with an unknown species that amounted to around 30% of the total arsenic. The EEs were again in the low 30% range.

Salmon

The speciation results for some salmon samples are shown in Table 4. The canned samples were purchased in Vancouver: their contents came from Canada, can 1, and the USA, can 2.

Arsenobetaine was by far the major species present in the methanol/water extracts and there was no indication of the unknown species reported by Lawrence et al. (1986). The low and variable EEs should be noted: consequently most of the arsenic in salmon remains unidentified. The inorganic arsenic found in base extracts (95°C) of Japanese salmon was 0.01 μg/g (fw), 1.4% of the total arsenic 0.7 μg/g (fw) (Mohri et al., 1990): the EE was

TABLE 4

Speciation of salmon samples (ppm, dw)

Sample	[As]total	AsB	Other species	%EE
Fresh salmon	1.40	0.29	As(III), DMA	31
Can 1	1.13	0.12	MMA	15
Can 2	1.88	0.12	MMA	8

higher at 86%. The arsenobetaine content of these extracts can be taken as 76% if it is assumed that the trimethylarsenic species present in the hydrolysate originates from arsenobetaine.

The brine from the canned salmon was also rich in arsenobetaine. For example, freeze-drying the brine from can 1 gave 7.24 g of solid (cf. 54.9 g of freeze-dried muscle from the same can) and 84% of the extractable arsenic was arsenobetaine. This amounts to about 10% of the arsenobetaine found in the muscle. MMA was the only other arsenical identified in the brine extract. Arsenobetaine was the major extractable species in the salmon after cooking.

Velez et al. (1995) found about 3.5 μg/g (fw) of arsenic in fresh salmon in studies that were restricted to AsB determinations. They also reported on the migration of AsB from the salmon muscle to the brine in canned products (Velez et al., 1997).

CONCLUSIONS

Arsenobetaine is generally believed to be harmless to humans and consequently its presence as the major arsenical in marine fish and to a lesser extent marine shellfish is not regarded as a health hazard. However, other arsenic species dominate in the fresh-water environment, so each food source should be evaluated in terms of speciation and health risk. Little is known about the arsenic speciation in salmon, a principal component of many diets.

ACKNOWLEDGEMENTS

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Arsenic Exposure and Health Effects IV
W.R. Chappell, C.O. Abernathy and R.L. Calderon (Editors)
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Determination of Organoarsenic Compounds in Finishing Chicken Feed and Chicken Litter by HPLC—ICP—MS

M. Pavkov, W. Goessler

ABSTRACT

Phenylarsonic compounds are used as feed additives for the control of *cecal coccidiosis* in poultry, swine and other domestic animals. Their use as growth promoters results in the growing interest in understanding their safety and toxicity during and after their use. A HPLC–ICP–MS was used for the determination and quantification of phenylarsonic acids in samples of finishing chicken feed, composted and uncomposted chicken litter from Texas, USA. The samples were microwave digested and measured for the total arsenic. The results obtained were the following (all for wet mass): 160 ± 14 μg As/kg in finishing chicken feed, 17, 300 ± 830 μg As/kg in composted chicken litter and 18, 500 ± 600 μg As/kg in uncomposted litter. As the frozen samples were extracted with Milli-Q water and chromatographed on a Hamilton PRP-X100 strong anion-exchange column with 50 mM $(NH_4)_2CO_3$ (2% MeOH v/v) at pH 11.0, in finishing chicken feed 3-nitro-4-hydroxyphenylarsonic acid (110 ± 16 μg As/kg wet mass) was found. The concentrations for the arsenic and arsenous acid were, respectively, 15.5 ± 2.2 μg As/kg and 10.1 ± 0.3 μg As/kg wet mass. In composted chicken litter no phenylarsonic acids were present. Arsenic acid accounted for 12800 ± 500 μg As/kg wet mass for water extractable arsenic. In uncomposted chicken litter phenylarsonic acids were found in the following concentrations: 20 ± 1 μg As/kg 4-aminophenylarsonic acid, 2900 ± 170 μg As/kg 4-hydroxyphenylarsonic acid, 200 ± 10 μg As/kg phenylarsonic acid, 4800 ± 310 μg As/kg 3-nitro-4-hydroxyphenylarsonic acid and 600 ± 30 μg As/kg of an unknown arsenic compound. The sum of total concentrations of arsenous-, arsenic-, methylarsonic- and dimethylarsinic acid was 1980 μg As/kg wet mass.

Keywords: phenylarsonic acids; chicken feed; chicken litter; anion-exchange chromatography; HPLC–ICP–MS

INTRODUCTION

For over 200 years, arsenic compounds have been used as therapeutic agents. Modern work on arsenical drugs started at the beginning of 20th century, when H.W. Thomas demonstrated that Atoxyl (sodium hydrogen 4-aminophenylarsonate), the first known aromatic arsenical, could cure experimental *trypanosomiasis* (Campell, 1964).

Differences in the disease-controlling and growth-promoting effects of different phenylarsonic acids result from the variation of substituents on the aromatic ring (Morehouse and Mayfield, 1946). These findings were further investigated at the beginning of the second half of the 20th century when evidence was presented that phenylarsonic acid and 3-nitro-4-hydroxyphenylarsonic acid, used as feed additives, could affect growth of broiler chickens (Carlson et al., 1954; Foster, 1972).

Following further investigations, it was shown that a number of phenylarsonic compounds control *cecal coccidiosis* in poultry, swine and other domestic animals. They also act as growth promoters, providing improved feed conversion and efficiency, better feathering, increased egg production and pigmentation (Anderson, 1983). *Coccidiosis* is a significant parasitic disease in all areas where livestock production is an important enterprise. Most domestic farm animals are susceptible to infection by coccidian parasites, and heavy losses are often incurred with animals raised in confinement.

Approved animal feed additives for both the poultry and swine are 3-nitro-4-hydroxyphenylarsonic acid (3-NHPA, roxarsone) and 4-aminophenylarsonic acid (4-APA). 4-nitrophenylarsonic acid (4-NPA), 4-ureidophenylarsonic acid (4-UPA) and phenylarsonic acid (PA) have also been used for such purposes and with the exception of 4-UPA, are still in use today (Fig. 1). Nowadays, questions regarding the efficacy, safety and toxicity, during and after the use of growth promoters are becoming more emphasized especially because the use of phenylarsonic acids as feed additives is allowed in the USA and some other countries but is not allowed within the European Union (Chiou et al., 1997). Therefore, different analytical methods have been developed for the identification and quantification of phenylarsonic compounds.

The Association of Official Analytical Chemists has developed three official methods for the determination of roxarsone (3-NHPA) in animal tissues (AOAC, 1995). Tech-

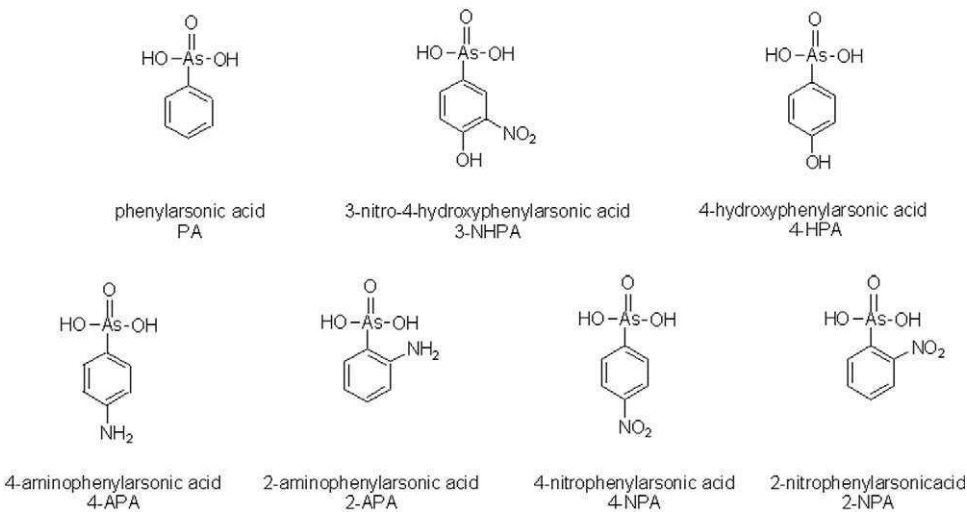

Fig. 1. Structures of investigated phenylarsonic acids.

niques like thin-layer chromatography (Morrison, 1968), non-suppressed ion-chromatography with anion-exchange column (Hirayama and Kuwamoto, 1988), μHPLC–ES–MS–MS (Pergantis et al., 1997) and μHPLC–ICP–MS (Pergantis et al., 1995) have been used for separation of different phenylarsonic and organic arsenic compounds.

The method developed by Pavkov and Goessler (2001) consisted of a HPLC–ICP–MS system with a HAMILTON PRP-X100 anion-exchange column. The separation of seven phenylarsonic acids was achieved within 25 min, but the 2-APA and 2-NPA were not resolved.

In this work we report on the determination of phenylarsonic compounds in chicken feed, uncomposted and composted chicken litter because of the growing interest in phenylarsonic acids during and after their use.

EXPERIMENTAL

Reagents, Standards and Mobile Phase

Standard solutions of 1000 mg (As)/l of the following compounds were prepared with Milli-Q (18.2 Ω cm) water: arsenous acid (As(III)) from $NaAsO_2$ and arsenic acid (As(V)) from $Na_2HAsO_4 \cdot 7H_2O$ were purchased from Merck; methylarsonic acid (MA) and dimethylarsinic acid (DMA) were gifts from the Vineland Chemical Company (Vineland, NJ).

Standard solutions of 1000 mg As/l of each of the following phenylarsonic acids were prepared with Milli-Q water to 100 ml each by dissolving 269.3 mg phenylarsonic acid (synthesized according to published procedures (Modro and Piekos, 1972), 350.6 mg 3-nitro-4-hydroxyphenylarsonic acid (Merck, Germany), 289.3 mg 2-aminophenylarsonic acid (Merck, Germany), 289.3 mg 4-aminophenylarsonic acid (Aldrich, Germany), 330.6 mg 4-nitrophenylarsonic acid (synthesized according to published procedures (Modro and Piekos, 1972), 330.6 mg 2-nitrophenylarsonic acid (Fluka, Switzerland) and 292.0 mg 4-hydroxyphenylarsonic acid (TCI, Japan).

The mobile phase used for identification and quantification of phenylarsonic acids was prepared by dissolving 4.80 g (50 mmol) ammonium carbonate (p.a., Merck, Germany) in Milli-Q water, adding 20 ml MeOH and filling to 1000 ml with Milli-Q water. The pH of the mobile phase was adjusted to pH 11.0 with aqueous ammonia (Suprapure, Merck, Germany).

The mobile phase used for identification and quantification of As(III), MA, DMA and As(V) was prepared by dissolving 2.30 g (20 mmol) ammonium dihydrogen phosphate (p.a., Merck, Germany) in Milli-Q water, adding 20 ml MeOH and filling to 1000 ml with Milli-Q water. The pH of the buffer was adjusted to pH 6.0 with aqueous ammonia (Suprapure, Merck, Germany) (Kuhnelt et al., 1997).

Methanol used as organic modifier was of the p.a. grade and product of Fluka, Switzerland.

Sample Preparation for the Chromatography

Frozen samples of finishing chicken feed, composted and uncomposted chicken litter from Texas, USA, were extracted with Milli-Q water. Samples of ~5.0 g were weighed to 0.001 g into 50 ml polyethylene tubes and Milli-Q water was added to the mark. Extraction of arsenic compounds was performed by sonication in an ultrasonic bath for 20 min after which the extracts were centrifuged at 2500 rpm for 10 min. The supernatants were filtered through 0.2 μm cellulose filters (Millipore, Bedford, MA, USA) and aliquots of 50 μl were chromatographed on the HPLC system.

Chromatographic System

The high-performance liquid chromatography system consisted of a Hewlett-Packard 1100 Series System (Hewlett-Packard, Waldbronn, Germany) — quaternary pump, auto sampler,

vacuum degausser, Rheodyne six-port injection valve and a 100 μl injection loop. The system was interfaced with an HP 4500 inductively coupled plasma mass spectrometry (ICP–MS) using a 1/16 inch PEEK (polyetheretherketon) capillary tubing.

The separations were performed on a Hamilton PRP-X100 (Hamilton Company, Nevada, USA), anion-exchange column (4.1 mm ID × 25 cm; spherical 10-μm particles of styrene–divinylbenzene copolymer with trimethylammonium exchange sites; stable between pH 1 and 13; exchange capacity 0.19 meq/g). A guard cartridge (Hamilton PRP-X 100) filled with the same stationary phase protected the analytical column. The flow-rate was 1.5 ml/min and the column temperature was 50ºC.

The ion intensities at m/z 75 (^{75}As) and m/z 77 ($^{40}Ar^{37}Cl$, ^{77}Se) were monitored using the ICP–MS. For data evaluation, chromatographic Software G1824C Version C.01.00 was used.

Operating conditions of the ICP–MS system are described in Pavkov and Goessler (2001).

Determination of Total Arsenic in the Samples

Aliquots of the samples (~0.3 g) were weighed to 0.001 g into Teflon digestion vessels. Concentrated nitric acid (5.0 ml) and 30% hydrogen peroxide (1.0 ml, Suprapure, Merck) were added to each vessel. The vessels were closed, secured in the rotor and placed in the microwave oven (MLS Ethos 1600 Microwave System, MLS, Leutkirch, Germany). In the first step of the digestion program temperature was raised to 85ºC in 2 min, to 145ºC within the next 5 min and finally to 210ºC in 3 min. At 210ºC samples were digested for 10 min. After the cooling period, the digests were transferred quantitatively into 50 ml polyethylene tubes and filled with Milli-Q water to the mark. The samples were further diluted and In as internal standard was added for measurement. The total arsenic concentrations were determined with a VG PlasmaQuad 2 Turbo Plus ICP–MS (VG Elemental, Winsford, UK).

RESULTS

Determination of Phenylarsonic Compounds in Finishing Chicken Feed, Composted and Uncomposted Chicken Litter

In order to determine the content of phenylarsonic compounds, calibration curves for the HPLC–ICP–MS measurements were obtained by injecting 50 μl of standard solutions containing 2.00, 5.00, 20.0, 50.0 and 100 μg As/l of each of the six phenylarsonic acids. Since the 2-APA and 2-NPA have co-eluted, only one of those compounds, namely 2-APA, was chosen for the mixture of standard aromatic arsenic compounds (Fig. 2).

The chromatographic system with $(NH_4)_2CO_3$ as the mobile phase did not allow the separation of DMA (1.79 min), As(III) (1.98 min), MA (2.40 min) and As(V) (2.64 min). Trimethylarsine oxide (1.61 min), tetramethylarsonium cation (1.66 min), arsenobetaine (1.92 min) and arsenocholine (1.94 min) eluted with the front.

A sample of finishing chicken feed was injected with 50 μl injection volume, whilst samples of composted and uncomposted chicken litter each were further diluted with water (1 + 9 v/v) prior to the injection (Figs. 3 and 4).

Phenylarsonic acids were quantified based on peak areas with external calibration curves. The presence of aromatic arsenic acids in finishing chicken feed, composted and uncomposted chicken litter was confirmed by spiking the extracts with standard solutions of phenylarsonic acids. The concentrations found in investigated samples are presented in Table 1.

As expected, the major arsenic compound in finishing chicken feed was roxarsone (3-NHPA) found in the amount of 110 ± 16 μg As/kg wet mass. In uncomposted chicken litter 4-APA (20 ± 1 μg As/kg wet mass), 4-HPA (2900 ± 170 μg As/kg wet mass) —

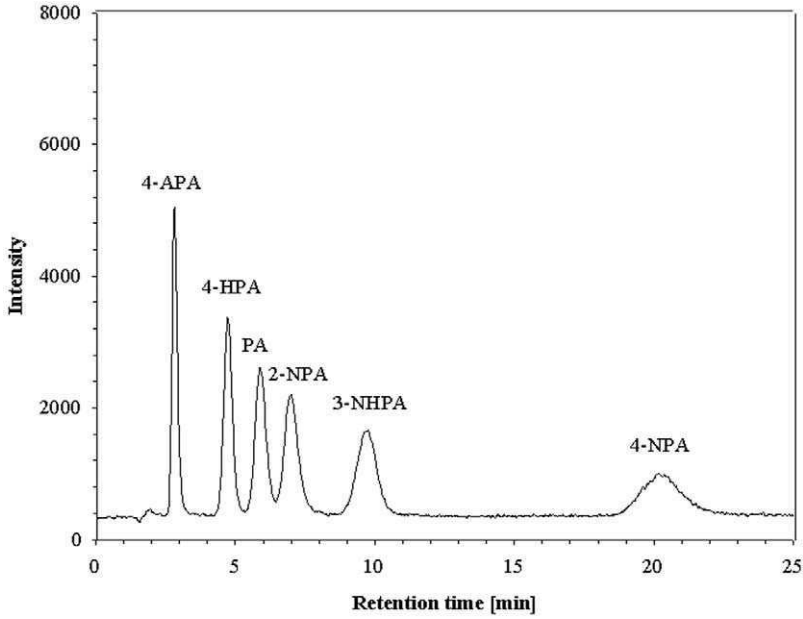

Fig. 2. Chromatogram of six standard phenylarsonic compounds. A 0.4 ng of each component (as arsenic) was injected. Hamilton PRP-X 100 anion-exchange column. Flow rate 1.5 ml/min at 50ºC, 50 mM ammonium carbonate at pH 11 with 2% MeOH (v/v). Volume injected 50 μl.

TABLE 1

Arsenic concentrations (μg As per kg wet mass) in finishing chicken feed, composted and uncomposted chicken litter (mean ± standard deviation ($n = 3$)

	Finishing chicken feed	Uncomposted chicken litter	Composted chicken litter
4-APA	<5	20 ± 1	<5
4-HPA	<5	2900 ± 170	<5
PA	<5	200 ± 10	<5
Unknown	<5	600 ± 30	<5
3-NHPA	110 ± 16	4800 ± 310	<5
4-NPA	<5	<5	<5
As(III)	10.1 ± 0.3	360 ± 10	110 ± 5
DMA	<5	470 ± 12	490 ± 15
MA	<5	150 ± 8	350 ± 8
As(V)	15.5 ± 2.2	1000 ± 100	12800 ± 500
Sum of arsenic compounds	136 ± 16	10500 ± 360	13800 ± 650
Total arsenic	160 ± 14	18500 ± 600	17300 ± 830
Water extractable arsenic	85%	57%	80%

the most probable degradation product, PA (200 ± 10 μg As/kg wet mass), and 3-NHPA (4800 ± 310 μg As/kg wet mass) were detected along with an unknown arsenic metabolite quantified with 3-NHPA external calibration curve. After composting, no phenylarsonic acids were detected in the litter.

As(V) and 4-APA could not be resolved with $(NH_4)_2CO_3$ as the mobile phase. There-

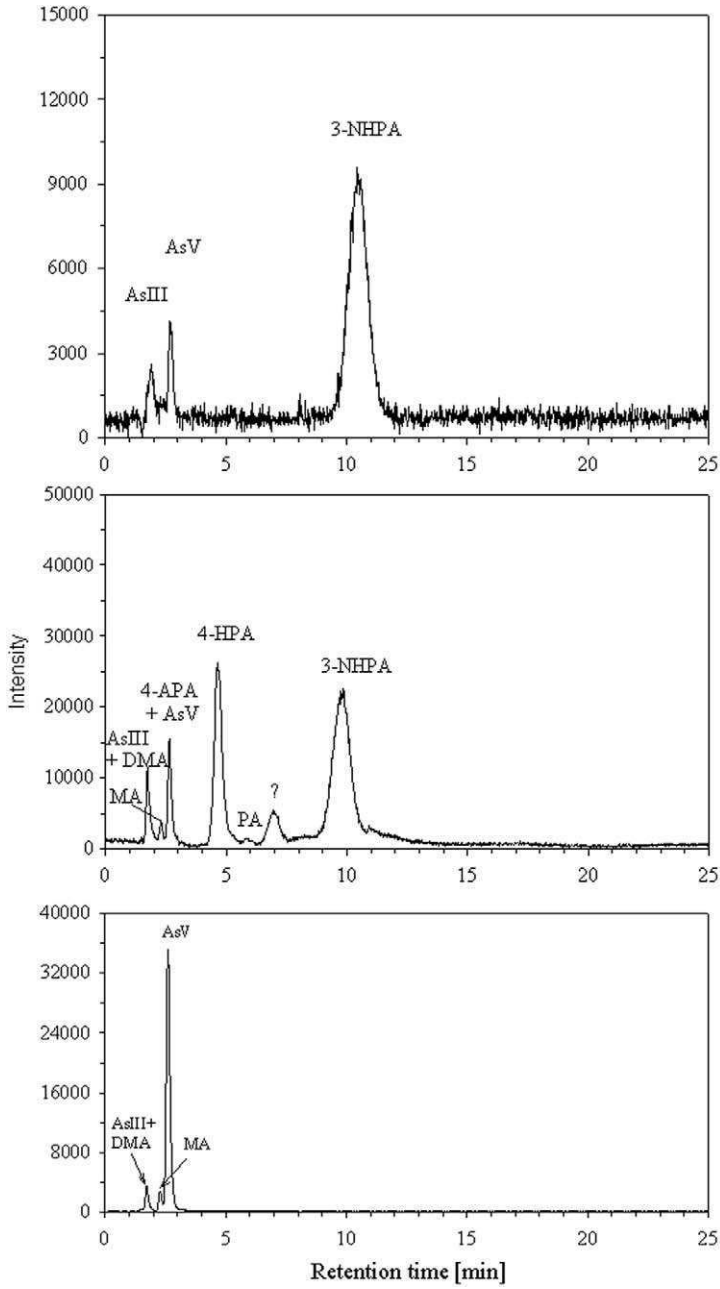

Fig. 3. Chromatograms on Hamilton PRP-100 anion-exchange column, 50 mM ammonium carbonate at pH 11 with 2% MeOH (v/v). Flow rate 1.5 ml/min at 50°C. Volume injected 50 μl. (A) Chicken feed water extract. (B) Composted chicken litter water extract, 5 μl composted chicken litter and 45 μl water. (C) Uncomposted chicken litter water extract, 5 μl uncomposted chicken litter and 45 μl water.

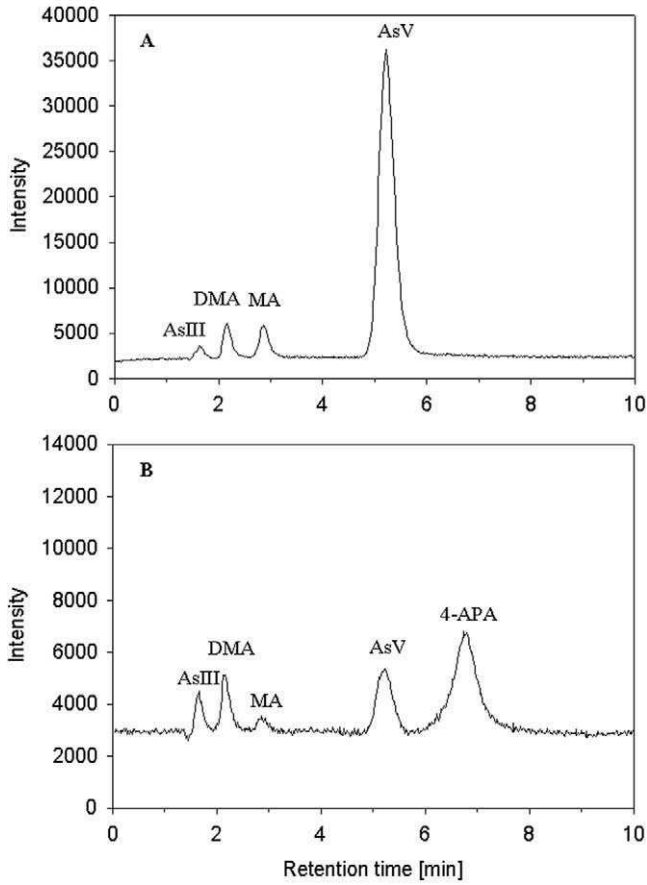

Fig. 4. Chromatograms on Hamilton PRP-X 100 anion-exchange column, 20 mM ammonium dihydrogen phosphate at pH 6.0 with 2% MeOH (v/v). Flow rate 1.5 ml/min at 25ºC. Volume injected 50 μl. (A) Composted chicken litter water extract, 5 μl composted chicken litter and 45 μl water. (B) Uncomposted chicken litter water extract, 5 μl uncomposted chicken litter and 45 μl water.

fore, a 20 mM ammonium dihydrogen phosphate solution at pH 6.0 with 2% MeOH (v/v) as mobile phase was used for the determination of the 4-APA. After the subtraction of the area for As(V) obtained with $NH_4H_2PO_4$ as the mobile phase from the area for the not resolved 4-APA and As(V) obtained with $(NH_4)_2CO_3$ as the mobile phase, the concentration of 4-APA was calculated. When using $NH_4H_2PO_4$ as the mobile phase the same elution order of the phenylarsonic acids was observed as with 50 mM ammonium carbonate as the mobile phase and 2-APA and 2-NPA were separated, even though the separation lasted for more than 60 min. With $(NH_4)_2CO_3$ as the mobile phase, the two compounds were not resolved and therefore it was not possible to say which one of them was found in the sample of uncomposted chicken litter. After chromatographing the samples with $NH_4H_2PO_4$ as the mobile phase, it was noticed that none of the peaks had the same retention time as 2-NPA or 2-APA which brought us to the conclusion that none of the two substances were present in the uncomposted chicken litter sample and the peak was identified as unknown.

Determination of Inorganic and Aliphatic Organic Arsenic Compounds

The more toxic arsenic compounds (As(III), As(V), DMA, MA) and possible degradation products, eluted almost with the solvent front and were not clearly separated with ammonium carbonate as mobile phase. Thus an additional chromatographic run with an aqueous 20 mM $NH_4H_2PO_4$ at pH 6.0 with 2% MeOH was performed on the samples.

The amounts of inorganic and aliphatic organic arsenic compounds found in each sample are presented in Table 1.

Inorganic and aliphatic organic arsenic compounds were quantified based on peak area upon spiking and external calibration curves. The sum of As(III) and As(V) found in finishing chicken feed was in a total amount of 25.6 μg As/kg wet mass. Both the composted and uncomposted chicken litter contained As(III), MA, DMA and As(V). The amount of As(V) determined in composted chicken litter (12800 ± 500 μg As/kg wet mass) was almost twelve times higher than the amount of As(V) found in uncomposted chicken litter (1000 ± 100 μg As/kg wet mass).

Total Arsenic Concentrations in the Samples

The concentration of the total arsenic after the microwave digestion was measured by ICP–MS and the results obtained were compared with those acquired with HPLC–ICP–MS after the chromatographic separation of aqueous extracts (Table 1).

After extraction of arsenic compounds from the samples with water, recovery of more than 50% of arsenic compounds was obtained. In general, phenylarsonic acids are spatially or good soluble in water (Cadogan et al., 1996a,b,c). Some of them, like 3-NHPA, are very soluble in MeOH and spatially soluble in water. For further investigations, water/methanol extraction might be used for effective extraction of phenylarsonic acids from samples.

Frozen samples were dried in an oven at 80ºC for 36 h. For finishing chicken feed, the water loss was 13.0%. Uncomposted and composted chicken litter lost 26.0% and 31.7% water, respectively.

DISCUSSION

The HPLC–ICP–MS method used in this paper allowed the identification and quantification of phenylarsonic acids in water extracts of finishing chicken feed, composted and uncomposted chicken litter. It was noticed that 3-NHPA, the phenylarsonic acid used as a drug against *cecal coccidiosis* and a growth promoter for chicken is excreted partially unchanged from the chicken body as determined in uncomposted chicken litter. It seems that phenylarsonic acids have a different metabolic pathway from that of aliphatic organic and inorganic arsenicals. Bahri and Romdane (1991) stated in their work about arsenic poisoning in livestock that phenylarsonic acids do not react with sulfhydryl groups as do trivalent inorganic and aliphatic arsenic compounds. The exact mechanism of the reaction of phenylarsonic acids inside the animal body has not yet been clarified. Nevertheless, the same authors stated that phenylarsonic compounds are poorly absorbed in the intestinal track and are largely excreted in feces.

Our results show that after composting the chicken litter tract 3-NHPA is most probably biotransformed by microorganisms to MA, DMA, As(V) and As(III).

Morrison (1969) studied the effect of fertilizing soil with poultry litter containing organoarsenical feed additives on the arsenic content in soil, crops grown on that soil, and drainage water passing through that soil. Contrary to our results, he found that the arsenic content of the soil and groundwater was apparently unaffected by treatment of the soil with poultry litter. He estimated that 1 to 2 mg/kg arsenic will be widespread per acre per year when chicken litter is used as fertilizer based upon a use rate of 4 to 6 tons of poultry litter per acre. Nowadays, it is estimated that approximately 10^6 kg per year (10^3 times more than in 1969) of roxarsone and its degradation products are introduced into the environment from the disposal of poultry litter onto agricultural fields near the chicken houses which can result in *localized arsenic pollution* (Wershaw et al., 1999).

Assuming that poultry litter is spread at a rate of approximately 15 m^3/ha (1 $m^3 \approx 500$ kg) on the fields with a total arsenic concentration of ~20 mg As/kg (most of the arsenic

already transformed to arsenate), this adds a total amount of ~0.2 kg/ha of arsenic in the environment. With this amount of arsenic, 2×10^4 m^3 of drinking water can be polluted to a final concentration of 10 μg As/l.

Food and Drug Administration regulations require that feeds containing phenylarsonic compounds must be withdrawn from poultry five days before slaughter (US National Academy of Sciences, 1977). Arsenic concentration in eggs and chicken tissues used by humans is limited to 0.5 mg/kg by FDA. Daghir and Hariri (1977) concluded in their study that the longer the chicken received the drug (3-NHPA), the less arsenic they transferred to their eggs. That indicates that they have developed a certain tolerance towards the drug since after repeated exposure a given dose of 3-NHPA produced a decreased effect.

As stated before, the broilers fed with 3-NHPA might have developed a certain tolerance towards the drug and might, by the time, in next generations of the broiler chickens treated by the same drug, develop a specific metabolic pathway for detoxification of phenylarsonic acids or become resistant to drugs containing arsenicals due to their overuse (Mellon, 2000).

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Arsenic Exposure and Health Effects IV
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Individual Susceptibility to Arseniasis

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ABSTRACT

ong-term exposure to ingested inorganic arsenic has been associated with an increased risk of various cancers and atherosclerotic diseases in a dose–response relationship. As only a small proportion of exposed subjects are affected with these diseases, there seems to exist an individual susceptibility to arseniasis. A series of studies have been carried out to elucidate both acquired and genetic susceptibility to arseniasis in the endemic areas of southwestern and northeastern Taiwan. Undernourishment status, indicated by long-term consumption of sweet potato and low serum level of carotenes, has been found to increase the risk of arsenic-induced peripheral vascular disease, ischemic heart disease and skin cancer. Poor inorganic arsenic methylation capability, indicated by high level of inorganic arsenic or monomethylarsonic acid in total metabolites of inorganic arsenic in urine, is associated with arsenic-induced peripheral vascular disease, carotid atherosclerosis and skin cancer. There exists the familial aggregation of arsenic-induced skin cancer and peripheral vascular disease after adjustment for exposure to arsenic in drinking water. Elevated frequency of chromosome-type chromosome aberration in peripheral lymphocytes has been found to increase the risk of skin cancer and transitional cell carcinoma. Genetic polymorphisms of glutathione S-transferases M1, T1 and P1 and DNA repair enzymes XPD and XRCC1 are associated with the risk of arsenic-induced skin cancer, while the genetic polymorphisms of apolipoprotein E, glutathione S-transferase P1 and p53 are associated with the risk of carotid atherosclerosis. Gene–gene and gene–environment interactions involved in the arsenic-induced health hazards deserve further investigation.

Keywords: arsenic; cancer; cardiovascular disease; individual susceptibility

INTRODUCTION

Arsenic is a ubiquitous element in the crust of the earth. It is mainly transported in the environment by water. High arsenic concentrations in groundwater have been observed in central Argentina, northern Chile, north-central Mexico, parts of west, mid-west and northeast United States, Inner Mongolia, Xinjiang and Shanxi in China, West Bengal in India, Bangladesh, Hungary, Algeria and Taiwan (Pontius et al., 1994; Chen et al., 1999). There is a shortage of surface water available for drinking and cooking due to the global change, man-made pollution and population growth. More and more people in the world are using underground water for drinking and cooking. Arseniasis is thus becoming an emerging epidemic in Asia (Chen et al., 1999)

Arsenic has been used as a drug or poison for nearly 4000 years. The lethal effects of arsenic are well documented. The acute and subacute toxicity of arsenic involve many organ systems including gastrointestinal, dermal, nervous, renal, hepatic, hematopoietic, cardiovascular, respiratory and ophthalmic systems (Chen et al., 1997a). Long-term exposure to a non-lethal dose of arsenic has also been found to induce chronic health effects in these organ systems. Inorganic arsenic induces cancers of the skin and several internal organs without any specific organotropism (Chen et al., 1997b). The systemic involvement of arsenic toxicity may result from the generalized distribution of ingested and inhaled arsenic in the human body, and the direct toxic effect of inorganic arsenic without metabolic activation.

Long-term exposure to ingested arsenic in artesian well water has been found to induce blackfoot disease (BFD), a unique peripheral vascular disease that ends with dry gangrene and spontaneous amputation of affected extremities in southwestern Taiwan (Chen and Wu, 1962; Chi and Blackwell, 1967). There exists a dose–response relationship between arsenic concentration in drinking water and risk of BFD (Tseng, 1977). A biological gradient with cumulative arsenic exposure has also been observed in recent studies on subclinical peripheral vascular disease detected by Doppler ultrasonography (Tseng et al., 1996), ischemic heart disease (Chen et al., 1996), cerebral infarction (Chiou et al., 1997), and carotid atherosclerosis (Wang, 1999). Both diabetes mellitus and hypertension, two major risk factors for cardiovascular diseases, have been found to be associated with ingested arsenic in a dose–response relationship (Lai et al., 1994; Chen et al., 1995; Tseng et al., 2000).

Ingested arsenic through medicinal, environmental and occupational exposures has been well documented to induce skin cancer (Chen et al., 1997b). A striking dose–response relationship between ingested arsenic from drinking water and skin cancer risk has been observed in a study on 40,421 residents in southwestern Taiwan (Tseng et al., 1968). Based on this study, the lifetime risk of developing skin cancer due to the ingestion of 1 μg/kg per day inorganic arsenic has been estimated to be 3.0 and 2.1 per 1000 for males and females (U.S. Environmental Protection Agency, 1988).

Elevated cancer risk of various internal organs including the liver, lung, kidney, urinary bladder, prostate, nasal cavity, colon/rectum, hematolymphatic system, and brain and nervous system has been associated with the long-term exposure to arsenic through inhalation and ingestion (Chen et al., 1997b). Ecological studies carried out in Taiwan have shown a significant dose–response relationship between arsenic concentration in drinking water and cancer risk of the skin, lung, liver, kidney, urinary bladder, prostate and nasal cavity (Chen et al., 1988a; Chen and Wang, 1990). Analysis of the ecological data using the Armitage-Doll multistage model has shown that the cancer potential of ingested inorganic arsenic is compatible for lung, liver, kidney and urinary bladder (Chen et al., 1992). The lifetime risk of developing cancers of the liver, lung, bladder and kidney due to a dayly intake of 10 μg/kg of arsenic was estimated as 0.43%, 1.2%, 1.2% and 0.42%, respectively, for males, and 0.36%, 1.3%, 1.7% and 0.48%, respectively, for females. In a recent further analysis of the excess lifetime risk estimates for several variations of the generalized linear

model and for the multistage Weibull model, the current standard of 50 μg/l has been found to be associated with a substantial increased risk of cancer (Morales et al., 2000). This suggests the standard of 50 μg/l drinking water may not be sufficiently protective of public health.

Despite the significant biological gradient of health effects with arsenic exposure, only a small proportion of arsenic-exposed subjects are affected with BFD, skin cancer and other arsenic-induced disorders. Furthermore, some residents were affected with arseniasis at low exposure levels, some with high levels. There seems to exist an individual susceptibility to arseniasis. In this report, we review studies on acquired and genetic susceptibility to arseniasis carried out in the endemic areas of southwestern and northeastern Taiwan.

SUSCEPTIBILITY TO BLACKFOOT DISEASE

Arsenic-induced Skin Lesion

Among the residents aged 60 or more years in the BFD-endemic area, the prevalence of BFD was 2.0%, 3.2% and 6.1%, respectively, for those whose drinking water had arsenic concentrations of <300, 300–599 and ≥600 μg/l (Tseng, 1977). In this prevalence study, skin lesions including hyperpigmentation, hyperkeratosis and various cancers were found to coexist with BFD showing relative risks of 9.7, 14.0 and 62.4, respectively, compared with those without the lesions. Arsenic-induced skin lesion may be considered a long-term biomarker of cumulative arsenic exposure and/or individual susceptibility to arseniasis. The arsenic level in drinking water was not adjusted in the estimation of relative risks of developing BFD for various arsenic-induced skin lesions in the previous study. In a recent case-control study, an increased risk of BFD was observed among those who had skin hyperkeratosis and/or cancer after adjustment for duration of consuming high-arsenic artesian well water and other risk factors (Chen et al., 1988b). The multivariate-adjusted odds ratio was around three-fold.

Undernourishment

An earlier study on the diet of residents in the BFD-endemic area found that the diet commonly consumed was dominated by sweet potato, intakes of fresh vegetables and fruits were markedly low, and fish was the only notable source of animal protein (Yang and Blackwell, 1961). The diet was adequate with respect to calories, high in carbohydrates, low in protein and extremely low in fat, which might include a deficient intake of essential unsaturated fatty acids. The intake of indispensable amino acids appeared to be above the minimum requirements with the exception of methionine and tryptophan. Although no attempt was made to evaluate vitamin and mineral intakes in the study, it appeared likely that vitamin intake was marginal. The dietary deficiencies were considered to be one of several factors required to induce BFD in susceptible individuals. An early case--control study showed that BFD patients had a lower socioeconomic status than matched controls showing odds ratios of 3.3 and 4.5, respectively, for those who had an average and below-average socioeconomic status as compared with those with an above-average socioeconomic status (Chi and Blackwell, 1967). A recent case-control study found that BFD patients had a significantly lower intake of meat, eggs and vegetables than matched controls (Chen et al., 1988b). Consumption of sweet potato as food staple was associated with an increased risk of BFD showing an odds ratio around two-fold as compared with consumption of rice. In our recent study on 29 patients affected with peripheral vascular disease and 198 healthy controls in the BFD-endemic area, low serum levels of α- and β-carotene were associated with an increased risk of the disease showing odds ratios (lowest quartile versus others) of 2.9 and 2.4, respectively.

Inorganic Arsenic Methylation Capability

Ingested and inhaled inorganic arsenic is methylated into monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in humans. As MMA and DMA are less toxic than arsenite and arsenate, the methylation is considered a detoxification process of inorganic arsenic. The arsenic methylation capability of an individual may be reflected by the relative proportions of inorganic arsenic, MMA and DNA in urine measured by high-performance liquid chromatography (HPLC)–hydride generation–atomic absorption spectrometry. In our recent study on 29 patients affected with BFD and 289 healthy controls, a higher percentage of inorganic arsenic and a lower percentage of DMA in urine were observed in BFD patients than in healthy controls. The high percentage of inorganic arsenic in urine (>6.7%) was associated with a two-fold risk of BFD compared with the low percentage (≤6.7%) after adjustment for age, sex, and duration of consuming high-arsenic artesian well water and serum carotene level.

Family History of Blackfoot Disease

In an early prevalence survey, a significant familial aggregation of BFD was observed (Wu et al., 1961). More than 15% of BFD patients had family members affected with BFD, while the BFD prevalence of the surveyed population was only 2%. The familial aggregation of BFD may be attributable to common genes and/or environments shared by family members. No adjustment for drinking high-arsenic artesian water was made in this study. In a recent case-control study (Chen et al., 1988b), a much higher proportion of BFD patients (7.6%) had a family history of BFD among first-degree relatives than matched controls (0.7%). The odds ratio of developing BFD was around three-fold after multivariate adjustment for risk factors including the duration of consuming artesian well water and the arsenic-induced skin hyperpigmentation and/or cancer. Further elucidation of genetic susceptibility to BFD will clarify the underlying mechanism of this familial aggregation.

SUSCEPTIBILITY TO ISCHEMIC HEART DISEASE

Blackfoot Disease Status

In a cohort study of ischemic heart disease among residents in the BFD-endemic area, a significant biological gradient was observed between the incidence of ischemic heart disease and the cumulative arsenic exposure (Chen et al., 1996). There was also an increased incidence of ischemic heart disease for patients affected with BFD as compared with the unaffected. The status of BFD may be considered an indicator of long-term arsenic exposure, systemic atherosclerosis and/or susceptibility to arseniasis. The association between BFD and ischemic heart disease remained significant after adjustment for cumulative arsenic exposure and other risk factors, showing a multivariate-adjusted relative risk of 2.5.

Low Serum Carotene Levels

In a recent case-control study on ischemic heart disease in arseniasis-hyperendemic villages (Hsueh et al., 1998), serum samples of 74 patients and 193 matched healthy controls were tested for serum levels of micronutrients by HPLC. A significant reverse dose–response relationship with arsenic-induced ischemic heart disease was observed for serum levels of α- and β-carotene, but not for serum levels of retinal, lycopene and α-tocopherol. The odds ratio of developing ischemic heart disease for those with a serum α- and β-carotene level < 8.7 μg/dl and a duration of consuming artesian well water ≥13 years was around five-fold, compared with those that had a serum α- and β-carotene level ≥ 8.7 μg/dl and a duration of consuming artesian well water <13 years, after adjustment for age, sex, body mass index, ratio between total cholesterol and HDL cholesterol, and hypertension.

SUSCEPTIBILITY TO CAROTID ATHEROSCLEROSIS

Inorganic Arsenic Methylation Capability

In a recent study in the arseniasis-endemic area of northeastern Taiwan, a significant dose–response relationship between arsenic in drinking water and ultrasonography-detected carotid atherosclerosis was observed (Wang, 1999). For those who consumed water with an arsenic concentration > 50 μg/l, the multivariate-adjusted odds ratio of developing carotid atherosclerosis was around three-fold compared with those with an arsenic concentration in drinking water ≤ 50 μg/l. The inorganic arsenic methylation capability was found to be associated with the development of carotid atherosclerosis among residents who consumed high-arsenic drinking water (>50 μg/l), showing an age–sex-adjusted odds ratio of 1.7 for those with a percentage of inorganic arsenic in total inorganic arsenic metabolites in urine ≥ 8.1% as compared with those with a lower percentage of <8.1% (Y.L. Chou, 2000). But the association between inorganic arsenic methylation capability and carotid atherosclerosis was not observed among those who consumed water with a low arsenic concentration (≤50 μg/l).

Genetic Polymorphisms of Apolipoprotein E and Lipoprotein Lipase

In the same case-control study in northeastern Taiwan, the genetic polymorphism of apolipoprotein E was significantly associated with carotid atherosclerosis among those who consumed water with an arsenic concentration ≤ 50 μg/l (Y.L. Chou, 2000). A lower risk of carotid atherosclerosis was associated with genotypes containing ε2 of apolipoprotein E compared with genotypes without ε2. But no association was observed between carotid atherosclerosis and genetic polymorphism of apolipoprotein E among those who consumed water with an arsenic concentration > 50 μg/l. No association between genetic polymorphism of lipoprotein lipase and carotid atherosclerosis was observed either.

Genetic Polymorphisms of Glutathione S-transferases and p53 Codon 72

In a recent study in northeastern Taiwan, the prevalence of carotid atherosclerosis was significantly associated with the genetic polymorphism of glutathione S-transferase (GST) P1, but not with GST M1 and T1 (Wang, 1999). The odds ratio of developing carotid atherosclerosis was around two-fold for those who had variant genotypes of GST P1 (VV/WV) compared with those having a wild genotype (WW) after adjustment for arsenic concentration in drinking water, age, sex, hypertension, and genotypes of p53 codon 72. The genetic polymorphism of p53 codon 72 was also associated with the risk of carotid atherosclerosis. The multivariate-adjusted odds ratio of developing carotid atherosclerosis was 1.6 for those who had variant genotypes of p53 codon 72 (VV/WV) compared with those who had a wild genotype (WW).

SUSCEPTIBILITY TO SKIN CANCER

Undernourishment

Among the residents aged 60 or more years in the BFD-endemic area, the prevalence of skin cancer was 4.8%, 16.5% and 25.6%, respectively, for males whose drinking water had arsenic concentrations of <300, 300–599 and ≥600 μg/l (Tseng et al., 1968). The corresponding figures for females were 0.9%, 6.2% and 11.0%, respectively. However, the status of undernourishment in the development of arsenic-induced skin cancer was not examined in this study. In a recent survey in the BFD-hyperendemic villages, the prevalence of skin cancer was found to increase with the duration of consuming sweet potato in a dose–response relationship (Hsueh et al., 1995). The multivariate-adjusted odds ratios were 5.5 and 8.5, respectively, for those who consumed sweet potato for 10–19 and ≥20 years compared with those that had a consumption duration of <10 years. In another nested case-control study in BFD-hyperendemic villages, the incidence of arsenic-induced

skin cancer was found to increase with the decrease in serum β-carotene level (Hsueh et al., 1997). The odds ratio for those who had a serum β-carotene level in the lowest tertile was around eleven-fold as compared with those who had higher levels after adjustment for age, sex, cumulative arsenic exposure and arsenic methylation capability.

Liver dysfunction

In the prevalence survey in the BFD-endemic area, both hepatitis B virus (HBV) chronic infection and liver dysfunction indexed by an elevated serum level of alanine transaminase were associated with an increased prevalence of skin cancer (Hsueh et al., 1995). The multivariate-adjusted odds ratio was 6.6 for HBV surface antigen carriers with liver dysfunction compared with non-carriers with normal liver function. It was hypothesized that liver dysfunction may reduce the methylation of inorganic arsenic in the liver and thus increase the deposition of inorganic arsenic in the skin. The detailed mechanism needs further elucidation.

Inorganic Arsenic Methylation Capability

In the nested case-control study aimed to examine the association between arsenic methylation capability and skin cancer risk, a higher MMA percentage and a lower DMA percentage in the total urinary inorganic arsenic metabolites level was observed among skin cancer patients than among healthy controls (Hsueh et al., 1997). The multivariate-adjusted odds ratio for those that had a cumulative arsenic exposure of $\geq$20.0 mg/l per year and a percentage of MMA $>$ 26.7% was around 24-fold, as compared with those that had a cumulative arsenic exposure of $<$20.0 mg/l per year and a percentage of MMA $\leq$ 26.7%.

Family History of Skin Cancer

The prevalence of arsenic-induced skin cancer in BFD-endemic areas was higher for those who had a family history of skin cancer among first-degree relatives than for those without such history (Hsueh et al., 1995). The age–sex-adjusted odds ratio was as high as four-fold, but it was not statistically significant due to the small sample size in the study.

Genetic Polymorphism of Glutathione S-transferases and p53

In a recent nested case-control study, a significant association with arsenic-induced skin cancer was observed for the combination of three GST genotypes (Tseng, 1999). For those who had at least one null or variant genotype of GST M1, T1 or P1, the risk of developing skin cancer was around five-fold as compared with those with wild genotypes of all three GST. There was a significant interaction between cumulative arsenic exposure, GST genetic polymorphisms and arsenic methylation capability. For those who had a cumulative arsenic exposure $>$ 14 mg/l per year, a poor inorganic arsenic methylation capability, and at least one null or variant genotypes of three GST, the risk of developing skin cancer was around 15-fold as compared with those with a cumulative arsenic exposure $\leq$ 14 mg/l per year, a normal arsenic methylation capability, and wild genotypes of three GST. There was no association between skin cancer risk and genetic polymorphism of p53 codon 72.

Genetic Polymorphisms of DNA Repair Enzymes

In a most recent nested case-control study, arsenic-induced skin cancer was found to be associated with genetic polymorphisms of DNA repair enzymes XPD and XRCC1 (Lin, 2000). The age–sex-adjusted odds ratios of developing skin cancer were 2.0 and 1.7, respectively, for the AA/AC genotypes (versus CC genotype) of XPD exon 6 and the Arg/Arg genotype (versus Arg/His or His/His genotypes) of XRCC1 exon 9. There were significant interactions between genetic polymorphisms of GST and DNA repair enzymes in the determination of arsenic-induced skin cancer. Further studies on genetic polymorphisms of other enzymes involved in DNA repair, xenobiotic metabolism and cell

cycle regulation may provide better understanding of gene–gene and gene–environment interactions in arsenic-induced skin carcinogenesis.

SUSCEPTIBILITY TO TRANSITIONAL CELL CARCINOMA

Blackfoot Disease and Arsenic-induced Skin Cancer

In a cohort study on urinary bladder cancer, mainly transitional cell carcinoma (TCC), in the BFD-endemic area, an increased risk was observed among patients affected with BFD as compared with the unaffected (Chiou et al., 1995). The odds ratio of developing urinary bladder cancer was around four-fold after adjustment for age, sex, cigarette smoking and cumulative arsenic exposure. In our most recent cohort study on TCC in the BFD-endemic and non-endemic areas, an increased risk of developing TCC was observed among patients affected with skin cancer than the unaffected. The odds ratio was around five-fold after adjustment for age, sex, cigarette smoking and cumulative arsenic exposure.

Chromosomal Aberrations

In a nested case-control study in the BFD-endemic area, an increased cancer risk was significantly associated with the elevated frequency of chromosome-type aberrations in peripheral lymphocytes (Liou et al., 1999). Both skin cancer and transitional cell carcinoma (TCC) were two major cancers newly diagnosed during the follow-up period. The odds ratio of cancer risk in subjects with more than zero chromosome-type breaks was 5.0 as compared with those who had zero chromosomal breaks. The odds ratio was 11.0 for more than zero chromosome-type breaks plus exchanges, and 12.0 for a frequency of total chromosome-type aberration > 1.007%.

Genetic Polymorphisms of Xenobiotic Metabolism and DNA Repair Enzymes and p53

In our preliminary case-control study on TCC in the southwestern arseniasis-endemic area, no significant association with TCC was observed for genetic polymorphisms of GST M1, T1 and P1 and N-acetyltransferase 2. The genetic polymorphisms of DNA repair enzymes XRCC1, hMSH2 and ATM and p53 codon 72 were not associated with the risk of TCC either (W.L. Chou, 2000).

CONCLUSION

There are several susceptible factors associated with the development of arsenic-induced cancers and atherosclerotic diseases. They include undernourishment, inorganic arsenic methylation capability, and genetic polymorphisms of enzymes involved in xenobiotic metabolism, DNA repair and cell cycle regulation. The pathogenesis of arseniasis may involve gene–gene and gene–environment interactions. Further investigations are essential for better understanding of arsenic-induced carcinogenesis and atherogenesis.

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Arsenic Exposure and Health Effects IV
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Bayesian Model Averaging with Applications to the Risk Assessment for Arsenic in Drinking Water

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ABSTRACT

Model selection often presents a challenge in the risk assessment process, especially when biologically based models are not apparent or fully developed. In a recent dose–response assessment for arsenic in drinking water, risk estimates were found to highly depend on the choice of the model. Two models could fit the data equally well, based on a standard model fit criterion, yet yield quite different risk estimates. Bayesian model averaging takes into account model uncertainty, more appropriately explains overall uncertainty about risk estimates. The current analysis uses lung cancer mortality data from the southwest region of Taiwan where high concentrations of inorganic arsenic were found in the drinking water.

Keywords: Bayesian model averaging; benchmark dose; generalized linear model

INTRODUCTION

Arsenic is a metal found in rocks and minerals in the earth's crust. Various forms of arsenic have been associated with several adverse health effects such as internal cancer and skin cancer in humans from both inhalation and ingestion. The primary source of ingestion is through drinking water. Currently, the United States Environmental Protection Agency (EPA) is revisiting the decision to lower the maximum contaminant level (MCL) to 10 μg/l (Safe Drinking Water Act Amendments, 1996). The current level, set by EPA in 1975 in response to the 1974 SDWA, stands at 50 μg/l (NRC, 1999).

In 1988, EPA published a special report on ingested inorganic arsenic. This risk assessment was based on an epidemiologic study investigating the associations between arsenic in drinking water and skin cancer (Tseng et al., 1968). Several sources of uncertainty were identified, including applicability of the risk assessment to the U.S. population, the role of arsenic as an essential nutrient and the relevance of skin lesions as the basis for the risk assessment. Since appearance of this report, there has been evidence that arsenic ingestion causes cancers more fatal than skin cancer (NRC, 1999).

There has been a great effort in measuring the associations between death from several internal cancers and arsenic in drinking water. In northern Chile, increased bladder and lung cancer mortality were found to be associated with arsenic in drinking water (Smith et al., 1998). Increased bladder cancer mortality was also found in Argentina (Hopenhayn-Rich et al., 1996). Also, exposure to inorganic arsenic may be associated with increased risk of infant mortality (Hopenhayn-Rich et al., 2000).

In 1999, the National Academy of Sciences released a report that reviews arsenic toxicity data, presents an analysis of bladder cancer data from Taiwan and evaluates the EPA (1988) risk assessment for arsenic in drinking water (NRC, 1999). Several issues concerning the dose–response estimation for arsenic were discussed, including model selection, measurement error caused by the ecological nature of the data, and other sources of uncertainty. The risk estimation was based on the multistage Weibull (MSW) model, which was found to be sensitive to exposure concentration grouping and village deletions. Risk estimates based on Poisson models were explored as an alternative and found to be less sensitive. Confidence limits based on the MSW required bootstrapping or some approximation while limits based on the Poisson models were straight forward to calculate.

A subsequent analysis of the Taiwanese bladder cancer data (Morales et al., 2000) revealed further problems related to model choice. Several models were found to fit the data well, according to accepted methods of model comparison, yet yielded fairly different risk estimates. There is also high variability in estimates depending on whether a comparison population is used. Since the models were non-nested, the Akaike information criterion (AIC) was used for model selection instead of a likelihood ratio test. Exposure levels associated with 1% excess risk benchmark dose (BMD_{01}) were calculated. In the male bladder cancer analysis with no comparison population, estimates based on the best fitting models according to AIC ranged from 351 to 395 μg/l. When a comparison population was used, BMD_{01} estimates ranged from 21 to 156 μg/l.

This article serves as a follow up to investigate a method that should reduce the error associated with choosing one particular model. Bayesian model averaging (BMA) takes into account model uncertainty, which in turn minimizes the overall uncertainty of risk for potential death of bladder and lung cancers from arsenic exposure. Standard frequentist methods that ignore model uncertainty can underestimate uncertainty about the parameters, then in turn overestimating confidence in the model leading to risky decisions (Volinsky et al., 1997).

A considerable amount of research has been done to tackle the issue of model uncertainty using Bayesian methods. Some methods such as those discussed by Newton and Raftery (1994) and Chib (1995), compute Bayes factors by calculating the marginal likeli-

hood or the normalizing constant of the posterior distribution. Other methods use Markov chain Monte Carlo (MCMC) methods to simulate the posterior distribution (Carlin and Chib, 1995). BMA was also used to assess the risk of stroke using proportional hazard models (Volinsky et al., 1997). This presentation will focus on the methods of Carlin and Chib and Raftery in more detail and compare the results of both methods applied to the Taiwanese internal cancer data with arsenic exposure.

METHODS

Taiwanese Lung Cancer Data

Data used in this analysis were derived from a study in an arseniasis endemic area of Taiwan (Chen et al., 1988, 1992; Wu et al., 1989). Lung cancer mortality data (person-years at risk and number of deaths due to cancer) were collected from death certificates of residents of 42 villages in 5-year age increments for both males and females during the period from 1973 to 1986. Drinking water samples were collected from wells in the 42 villages.

Statistical Approach

For the purpose of dose–response modeling the number of deaths due to lung cancer (d) within each age group and village were assumed to follow a Poisson distribution with the parameter equal to the person-years at risk (PYR), multiplied by the hazard of dying ($h(x,t)$), i.e., $d \approx \text{Poisson}(\text{PYR} \cdot h(x,t))$. The lifetime risk of dying from lung cancer at exposure x, $\text{ldr}(x)$, can be expressed as a function of the parameters from the Poisson model,

$$\text{ldr}(x) \approx 1 - \sum_t q_t \exp\left(-5 \sum_{s<t} h(x,s) \right)$$

where $\sum_t$ denotes the sum over all 5-year age groups in the study and q_t is the probability of dying during the 5-year time interval indicated by t. The excess lifetime risk is the risk over background levels, $\text{edr}(x) = \text{ldr}(x) - \text{ldr}(0)$. The ultimate goal is to calculate the benchmark dose (BMD) or the dose associated with a specified level of excess risk. This level is usually 10%, according to EPA's guidelines (EPA, 1996), but for epidemiological data an excess risk of 10% occurs in relatively high doses, sometimes outside the observed range of the data. We considered both 1% and 5% excess risks, BMD_{01} and BMD_{05}, respectively. The lower 95% confidence limits are denoted by BMDL_{01} and BMDL_{05}, respectively.

It has been shown that choosing the most appropriate model is not a straightforward task. As with the Taiwanese bladder cancer data, we observed a wide range of risk estimates based on models with a similar fit according to AIC (Morales et al., 2000). The results did not seem satisfactory. Bayesian model averaging provides the framework for getting past the issue of model uncertainty. Posterior model probabilities weight the performance of each model considered in comparison with each other (probabilities sum to 1).

We investigate two methods for calculating posterior model probabilities. Raftery (1996), suggests an asymptotic approximation using Laplace's method for integrals. Carlin and Chib (1995) suggest a Markov chain Monte Carlo method. A set of 9 generalized linear models are considered. Each model contains a linear dose effect, a dose transformation (identity, $\log(1+x)$ or square root) and age effect (linear, quadratic or non-parametric). For example, one possible model uses an identity transformation on dose with a linear dose effect and a quadratic age effect. Models were fit without a baseline comparison population. Results from the analysis of the Taiwanese bladder cancer data showed that, for these data, including a baseline comparison population had a strong influence on the estimation

of risk (Morales et al., 2000). Therefore, in the current analysis, the shape of the curve at low doses will be determined only by the model.

Posterior probabilities based on the two methods were compared to other model fit criteria such as the AIC and Bayesian information criteria (BIC). Both statistics are a function of the log-likelihood function. The smaller value indicates the better fitting model. Averaged risk estimates were calculated based on a weighted average with weights equal to the posterior model probabilities.

RESULTS

Table 1 contains risk estimates based on each of the 9 models. Models are described by the dose transformation and age effect. BMD_{01} estimates range from 9 to 421 μg/l. Table 2 shows the model fit comparisons and posterior model probabilities based on the two methods: Carlin and Chib (CC) and Raftery. According to AIC, models 5 and 8 fit reasonably well with BMD_{01} estimates equal to 13 μg/l and 84 μg/l, respectively. Interestingly, the model fit criterion and the posterior probabilities are consistent in terms of which models they suggest fit well.

Table 3 contains 1% and 5% BMD estimates and lower 95% confidence limits for the averaged model based on both the Carlin and Chib and Raftery methods. The table also includes an estimate of the margin of exposure (MOE), which is the ratio of BMD_{01} to the

TABLE 1

Benchmark dose estimates (male lung cancer)

Model (transform., age)	BMD_{01} *	$BMDL_{01}$ *	BMD_{05} *	$BMDL_{05}$ *
1 (iden, lin)	293	237	1136	920
2 (iden, quad)	364	294	1345	1086
3 (iden, nonpar)	421	343	1502	1219
4 (log, lin)	9	6	220	156
5 (log, quad)	13	9	389	265
6 (log, nonpar)	18	12	582	397
7 (sqrt, lin)	55	41	720	528
8 (sqrt, quad)	84	62	981	714
9 (sqrt, nonpar)	111	84	1200	880

* Terms defined in text.

TABLE 2

Model fit comparison (male lung cancer)

Model (transform., age)	AIC *	BIC *	Raftery pM *	CC pM *
1 (iden, lin)	392.3500	405.2578	0.0000	0.0000
2 (iden, quad)	370.3580	387.5685	0.1278	0.1377
3 (iden, nonpar)	372.7534	398.5691	0.0382	0.0437
4 (log, lin)	391.5769	404.4848	0.0000	0.0000
5 (log, quad)	369.3735	386.5840	0.2598	0.2313
6 (log, nonpar)	371.6957	397.5114	0.0804	0.0851
7 (sqrt, lin)	390.4170	403.3248	0.0000	0.0000
8 (sqrt, quad)	368.3619	385.5724	0.3787	0.3711
9 (sqrt, nonpar)	370.7207	396.5364	0.1152	0.1310

* Terms defined in text.

TABLE 3

Risk estimates for lung cancer using 2 models

Average model [a]		Lung cancer	
		Male	Female
Raftery:	BMD_{01}	112	236
	$BMDL_{01}$	– [b]	133
	$MOE_{01}(50)$	2.2	4.7
	BMD_{05}	887	876
	$BMDL_{05}$	299	804
	$MOE_{05}(50)$	17.7	17.5
Carlin and Chib:	BMD_{01}	88	259
	$BMDL_{01}$	12	78
	$MOE_{01}(50)$	1.8	5.2
	BMD_{05}	994	893
	$BMDL_{05}$	345	711
	$MOE_{05}(50)$	19.9	17.9

[a] See text for explanation of terms.
[b] BMDL below range of data.

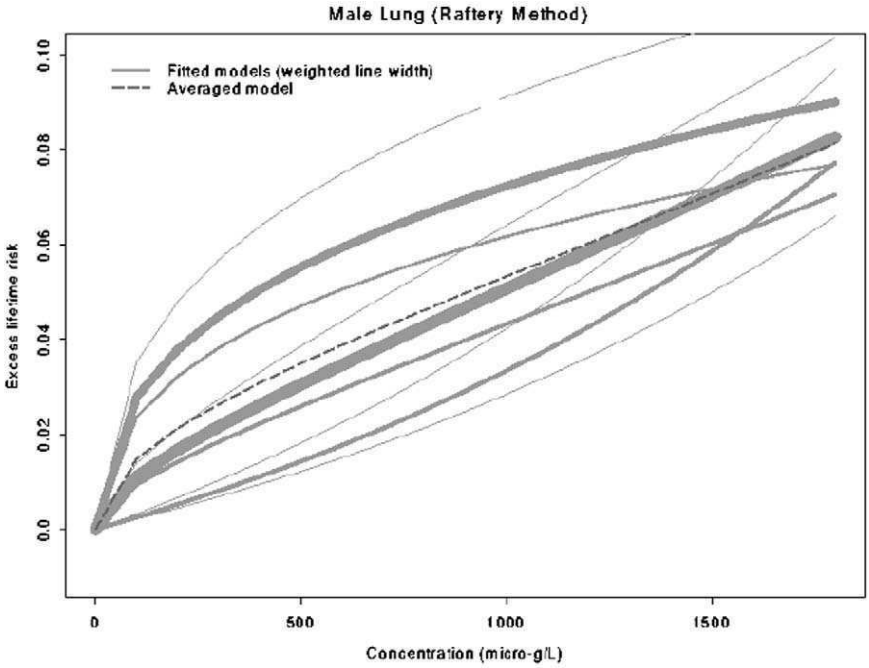

Fig. 1. Estimated excess lifetime risk for male lung cancer (Raftery's method).

environmental exposure of interest (50 μg/l). BMD_{01} estimates equal 112 μg/l and 88 μg/l. Fig. 1 gives a graphical representation. The solid lines represent the estimated excess lifetime risk based on the 9 models. The line thickness of the 9 models is proportional to the

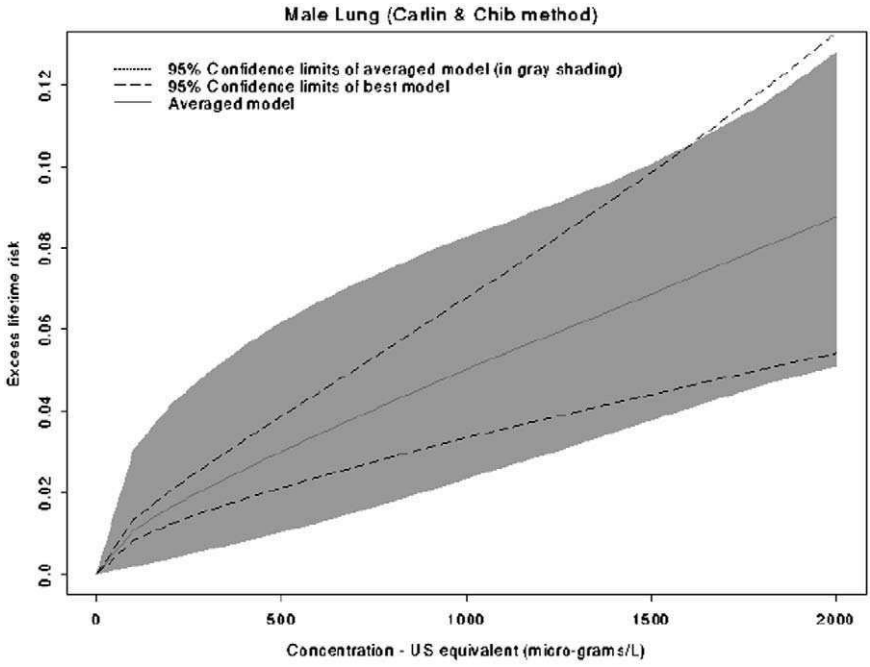

Fig. 2. Estimated excess lifetime risk for male lung cancer (Carlin & Chib method). Dashed line represents 95% confidence limits for the best model. The dotted lines filled in gray represent the 95% confidence limits for the averaged model.

posterior model probabilities. The dashed line is the averaged model based on Raftery's method. Fig. 2 shows graphical results from Carlin and Chib's method. The solid line represents the averaged excess lifetime risk curve with the 95% confidence limits shaded. For comparison, the 95% confidence limits for the best model are shown by the dashed lines.

DISCUSSION

We have provided an example of how Bayesian model averaging can be used to control for model uncertainty in the dose–response estimation process. BMD estimates were obtained without selecting a single model. Hoeting et al. (1999) suggest that "in theory, BMA provides better average predictive performance than any single model that could be selected." This method also provides a quantitative assessment of the impact of model selection.

The Carlin and Chib method is preferred over the Raftery method for a couple of reasons. Even though Raftery's method is computationally simpler, it uses an approximation. Also, risk estimates based on Raftery's method could possibly fall outside the range of the data. As observed in Table 3, the lower 95% confidence limit for BMD_{01} based on Raftery's method falls below zero. Whereas with the Carlin and Chib method, an estimated BMD inside the range of data is insured since estimates are based on percentiles of the posterior distribution.

As one would expect, the effect of model uncertainty is greater at low dose levels. In our example, the model uncertainty is best shown in Fig. 2. There is more variability at low doses between the 95% upper confidence limit for the 'best' model and the 95% upper confidence for the averaged model.

More work is needed to assess the robustness of Bayesian model averaging to the initial set of models and assessing goodness of fit. Also, there are still sources of uncertainty which have not been accounted for, including the potential measurement error induced by not having individual exposure measurements. More research is needed to quantitatively assess the impact of this and other sources of uncertainty on benchmark dose estimates.

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Epidemiological Study on Various Non-carcinomatous Manifestations of Chronic Arsenic Toxicity in a District of West Bengal

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ABSTRACT

Chronic arsenic toxicity due to drinking of arsenic (As) contaminated ground water has been a major environmental health problem in West Bengal. That chronic arsenicosis produces protean clinical manifestations has been reported by us earlier on the basis of study of a large number of hospitalized cases. Data of a cross-sectional epidemiological study in one of the affected districts, published previously, demonstrated that the prevalence of cutaneous manifestation was strongly related to As concentrations in water. Further data in regard to various other non-carcinogenic manifestations of chronic arsenicosis are being presented on the basis of the same epidemiological study. Out of a population of 7683 surveyed, 3467 and 4216 people consumed water containing As below and above 0.05 mg/l, respectively. Incidence of weakness, hepatomegaly, chronic lung disease, dyspepsia and neuropathy were found among 1.37, 2.99, 7.74, 31.81 and 2.73% in the former group against 4.99, 10.21, 11.68, 27.84 and 4.7% in the later group, respectively. Except dyspepsia, the incidences of the other features were found to be significantly higher in the high As exposed group compared to control population (drinking water As < 0.05 mg/l). The distribution of age, sex and As concentrations in water of the study population were correlated with the incidence of hepatomegaly and respiratory symptoms and weakness. A significant linear dose–response relationship was observed in regard to all those manifestations and As exposure. Arsenic concentrations in hair and nail were tested in 55 and 37 cases, respectively, among people showing features of dermatological manifestations of high As exposure in drinking water (>0.05 mg/l). Overall, incidence of elevated As concentrations in hair and nail was found in 81.82 and 62% of cases. The results of cross-sectional epidemiological study in West Bengal substantiated that chronic As toxicity in man produces protean non-carcinogenic manifestations, such as weakness, liver enlargement, chronic-lung disease and peripheral neuropathy. Determination of As concentrations in hair and nail does not help in the diagnosis of arsenicosis in all cases.

Keywords: chronic arsenicosis; arsenic and liver disease; arsenic and lung disease; arsenical neuropathy

INTRODUCTION

Since 1984 several reports of chronic arsenic toxicity due to drinking of arsenic-contaminated water in West Bengal have been published (Guha Mazumder et al., 1988, 1997, 1998, 1999; UNICEF, 1998; Santra et al., 1999). Incidence of arsenic contamination in subsoil water and the subsequent sufferings of people from chronic arsenic toxicity are increasing in eight districts of West Bengal, covering an area of 34,493 sq. km and the population at risk is about 6 million. On the basis of data available to date, it is believed that one of the largest arsenic calamities in the world is in West Bengal.

The early epidemiological studies were conducted to understand the gravity of the problem, but these studies were limited to a few arsenic-affected villages. To better understand the prevalence of chronic arsenic toxicity and the severity of morbidity caused by drinking arsenic-contaminated subsoil water in the population, an epidemiological study was carried out in one of the affected districts of West Bengal. Data on skin manifestations with individual dose of chronic As exposure have already been published in a previous report (Guha Mazumder et al., 1999). Here we report the clinical spectrum of various disease manifestations including the prevalence of liver affection and lung disease associated with chronic arsenic toxicity as observed in this study.

PATIENTS AND METHODS

The study was carried out on 7683 people (4093 females and 3590 males) out of a population of 150,457 in 57 villages of South 24 Parganas, an affected district of West Bengal, India. Water samples were collected from private and public tubewells used for drinking and cooking purposes by each recruited household. Arsenic concentrations were measured by flow-injection hydride generation atomic absorption spectrophotometry. A full history of water intake including source and duration of water intake was taken from the study population to ascertain an As-related clinical effect. Details of the methods used for the survey were described earlier (Guha Mazumder et al., 1999). A brief account of the methodology is described below.

INTERVIEW AND CLINICAL EXAMINATION

Each participant was asked questions about the various symptoms, socioeconomic status, the dietary habits, addiction, past history of major illness, and history of parasitic infestation. A thorough clinical examination of each participant was carried out taking special care to detect skin pigmentation and keratosis, and enlargement of the liver. Specific symptoms like weakness, abdominal pain or nausea (suggesting affection of the alimentary system), tingling and numbness (paresthesia, suggestive of nervous system disorder) were also recorded.

Patients diagnosed with liver enlargement were further interrogated regarding any past history of jaundice, parasite expulsion, history of taking alcohol or other hepato-toxic drugs. In regard to the respiratory system, if the participant did not volunteer any information concerning the presence of a respiratory problem, they were then specifically asked whether they had any cough or breathing difficulty. Crepitations were determined by auscultation and were defined as the presence of any chest sounds including rals or rhonchi. Participants were also asked if they were current or past smokers.

BLOOD SAMPLING

Blood samples from willing participants having hepatomegaly were collected for liver function tests (serum protein and alanine amino transferase (ALT)), and serodiagnostic

tests such as amoebic serology (by commercially available EIA kit), Hepatitis B surface antigen (HBsAg) by ELISA (Hepanostica, Organon Technica, Belgium), and Anti Hepatitis C Virus (HCV) by 3rd-generation ELISA kit (Hepanostica, Organon Technica, Belgium).

ARSENIC CONCENTRATIONS IN HAIR AND NAILS

Arsenic concentrations in hair and nail were tested in 55 and in 37 cases respectively showing skin manifestations of chronic arsenic toxicity. On the basis of analysis of hair and nail on 50 control Indian population (As concentrations in hair 0.41 ± 0.21 and in nail 0.95 ± 0.17 mg/kg) (Das et al., 1995) values above mean +2 SD, i.e., 0.85 mg and 1.20 mg/kg for hair and nail, respectively, were considered to be elevated in the affected subjects.

STATISTICAL ANALYSES

Prevalence of liver disease was stratified by age and calculated for each sex separately. The outcome was analyzed for participants who reported cough, shortness of breath, and weakness, and the presence of crepitations as recorded by the examining physician. To allow for direct comparisons without the distorting effects of age, the prevalence of each outcome was directly standardized to the age distribution of all study participants of the same sex. Each outcome was examined according to arsenic concentrations in the tubewell drinking water source used by each participant which were categorized according to arsenic concentrations as follows: <50, 50–199, 200–499, 500–799 and ≥ 800 μg/l.

Tests for trends in proportions using the midpoints of the exposure categories were based on the chi-square distribution (Breslow and Day, 1980). In view of uni-directional a priori hypotheses, one-sided *P*-values are presented for the test of trends.

Prevalence odds ratios (POR) were also calculated for each outcome comparing those with very high exposure to arsenic in drinking water (≥ 500 μg/l) with those with the lowest exposures (<50 μg/l). The Mantel–Haenszel method was used to adjust for age. We also stratified these analyses according to the presence or absence of arsenic-caused skin lesions (Guha Mazumder et al., 2000).

RESULTS

The result of this epidemiological survey in the district of South 24 Parganas showed that the inhabitants were traditionally dependent on ground water and the source was shallow tubewells, varying in depths from 21.3 to 305 m, some of which had been found to be contaminated with arsenic. The arsenic concentration in tubewell water in the villages ranged up to 3400 μg/l, but 88% of the participants with recorded As concentrations in water were exposed to levels less than 500 μg/l. Among 7683 people surveyed, 3467 people drank water containing arsenic less than 0.05 mg/l (maximum permissible limit of As in India) and 4216 people drank water containing high concentrations of arsenic (0.05 to 3.4 mg/l) (Table 1). The clinical presentations of the study population giving the incidence of pigmentation, keratosis, weakness, hepatomegaly, chronic lung disease, dyspepsia and neuropathy among both groups are given in Table 2. Except dyspepsia, the incidence of other features were found to be significantly higher in the As-exposed group compared to control population (drinking water ≤ 50 μg/l).

History of the duration of water intake was available from 3393 out of 3467 people who used water with As concentrations less than 0.05 mg/l. Out of these, 271 people drank water with As concentrations less than 0.01 mg/l and 3122 with concentrations of more than 0.01 but less than 0.05 mg/l. It can be seen from Table 3 that all 12 people who had pigmentation and the 4 people who had keratosis in the latter group had consumed their

TABLE 1

Population in the study area, number of participants in the study and number of tube-wells from where water samples were tested for arsenic in the study area

	High exposure region [a]	Low exposure region [b]	Total
Total population	53,313	97,144	150,457
No. of individuals examined	5,164	2,654	7,818
Individuals included for analysis	5,087	2,596	7,683
No. of tubewell water samples tested for As	575	87	662
As level < 50 μg/l	299	64	363
As level ≥ 50 μg/l	260	21	281

[a] No. of households in high-exposure region: 874.
[b] No. of households in low-exposure region: 467.

TABLE 2

Incidence of various clinical manifestations of chronic arsenicosis: epidemiology study in one of the affected districts of West Bengal (24 Parganas-South) 1995–1996

Symptoms	As level < 50 μg/l ($n = 3467$) (%; n value)	As level ≥ 50 μg/l ($n = 4216$) (%; n value)	P value
Pigmentation	0.34; 12	8.82; 372	<0.001
Keratosis	0.11; 4	3.64; 154	<0.001
Hepatomegaly	2.99; 104	10.21; 431	<0.001
Weakness	1.37; 48	4.99; 211	<0.001
Abdominal pain	31.81; 1103	27.84; 1174	NS
Nausea	0.31; 11	0.74; 31	<0.02
Lung disease	7.74; 269	11.68; 493	<0.001
Neuropathy	2.73; 95	4.70; 198	<0.001

TABLE 3

Cutaneous manifestations with low As dose exposure correlation with duration of water intake

	Pigmentation		Keratosis	
	<10 μg/l	≥10 to <50 μg/l	<10 μg/l	≥10 to <50 μg/l
Duration < 5 years total	19	348	19	348
Symptoms	0	0	0	0
Percentage	0	0	0	0
Duration 5 to <10 years	42	437	42	437
Symptoms	0	0	0	0
Percentage	0	0	0	0
Duration ≥ 10 years	210	2337	210	2337
Symptoms	0	12	0	4
Percentage	0	0.51	0	0.17

tubewell water for 10 years or more. On the other hand none had any skin manifestation who drank water with a concentration of As < 0.01 mg/l.

It can be seen from Table 2 that enlargement of the liver was found in 104 people with drinking water containing arsenic < 0.05 mg/l (low exposure group) compared to 431

people with drinking water containing arsenic 0.05 mg/l or more (high exposure group). The liver was firm and palpable, 2–6 cm below the costal arch in all the cases. Of the various etiological factors for hepatomegaly, e.g., history of chronic alcohol intake, past history of jaundice and round worm expulsion were found among 5 (4.8%), 26 (25%) and 29 (27.8%) participants in the former group while in 19 (4.4%), 55 (12.76%) and 132 (30.62%) subjects in the latter group, respectively. Blood could be tested for viral markers among 26 people belonging to the low exposure group and 143 people to high exposure group. HBsAg was found to be positive in 2 (7.69%) in the former group and 7 (4.82%) in the latter group. Anti-HCV was negative in all cases belonging to both the groups. Further, anti-amoebic antibody titre was found to be high (>1 : 125) in the blood of 6 (26.08%) out of 23 participants of the former group and 25 (25.25%) out of 99 participants of the latter group. Thus the significantly high incidence of hepatomegaly observed in the As-exposed group compared to control population could not be incremented to any of the common etiological factors associated with hepatomegaly in the tropics. Of the various parameters of liver function tests, serum albumin, globulin and ALT levels were done among 124 people, drinking high-As water (>0.05), having hepatomegaly without any known etiological factor (i.e., history of jaundice, HBsAg and HCV seropositivity and elevated titre of amoebic serology). Abnormal serum albumin level (<3.0 g/dl) was found in 4 cases (3.22%) and globulin (>3.5 g/dl) was found in 17 cases (13.71%), respectively. Elevation of serum ALT (>40 U/l) was found in 27 cases (21.77%).

The distribution of age, sex and concentrations of As in water of the study population has been published earlier (Guha Mazumder et al., 1998). Prevalence of hepatomegaly corresponding to various As dose exposures among males and females are plotted in Fig. 1. The prevalence of hepatomegaly was found to have a linear relationship proportional to increasing exposure of As in drinking water in both sexes ($P < 0.001$). The prevalence of

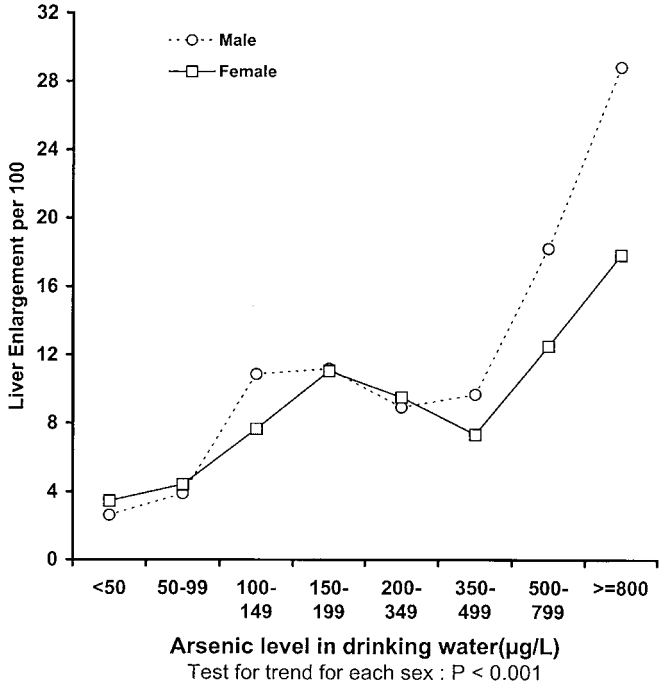

Fig. 1. Prevalence of liver enlargement per 100 in relation to arsenic exposure.

TABLE 4

Distribution of nonsmoking participants by age, sex and arsenic level in drinking water (μg/l) (Guha Mazumder et al., 2000)

Age group	Arsenic level (μg/l)					Total
	<50	50–199	200–499	500–799	≥800	
Females						
≤9	194	107	134	75	26	536
10–19	399	186	173	65	26	849
20–29	572	275	197	83	23	1150
30–39	304	172	119	44	15	654
40–49	168	78	55	28	9	338
50–59	156	74	43	29	11	313
≥60	94	52	41	9	6	202
All ages	1887	944	762	333	116	4042
Males						
≤9	220	156	128	81	28	613
10–19	313	166	144	62	29	714
20–29	292	140	96	50	20	598
30–39	147	84	79	38	15	363
40–49	78	58	45	14	5	200
50–59	65	31	35	18	8	157
≥60	82	48	31	12	4	177
All ages	1197	683	558	275	109	2822

hepatomegaly among females was 3.46% in the lower exposure category (<50 μg/l) while its incidence was 17.83% in the highest exposure category (≥800 μg/l). A stronger trend appeared in males, the prevalence increased from 2.62% in the lowest category to 28.81% in the highest exposure category. The prevalence was greater among males than females.

Results of pulmonary effects manifested by cough, crepitation and shortness of breath among nonsmokers were analyzed excluding 819 smokers because of their small numbers and potential confounding. Table 4 presents the age and sex distribution of all nonsmoking participants by arsenic concentrations in drinking water. Prevalence of cough and crepitation are shown in Figs. 2 and 3. The overall prevalence in females for each respiratory outcome was close to 2.5 per 100. Clear trends of increasing prevalence by arsenic concentration in water can be seen for cough ($P < 0.0001$) and crepitation ($P = 0.002$). Among males, the overall age-adjusted prevalence of cough (5.2 per 100) and crepitation (4.4 per 100) was nearly twice as high as among females but once again there were trends of increasing prevalence by arsenic concentration in water ($P = 0.001$ and $P = 0.04$, respectively). The prevalence of both cough and crepitation increased with increasing arsenic content in children of both sexes in the age range of 10–19 years (Guha Mazumder et al., 2000). However, the prevalence of shortness of breath in females showed a markedly nonlinear relationship ($P < 0.001$) peaking in the third exposure category (200–499 μg/l), but declining sharply thereafter to baseline concentrations. The overall age-adjusted prevalence for shortness of breath in males (3.6 per 100) was higher than in females (2.6 per 100) (Guha Mazumder et al., 2000). Table 5 gives prevalence odds ratios comparing the highest exposure category (≥500 μg/l) with the lowest exposure category (<50 μg/l). All prevalence odds ratios are elevated, but particularly so for shortness of breath among females and weakness in both sexes. Table 6 also presents prevalence odds ratios separately for those with and without arsenic-caused skin lesions (keratoses and/or pigmentation changes). Markedly increased prevalence odds ratios are seen for each outcome in both males and females with skin lesions.

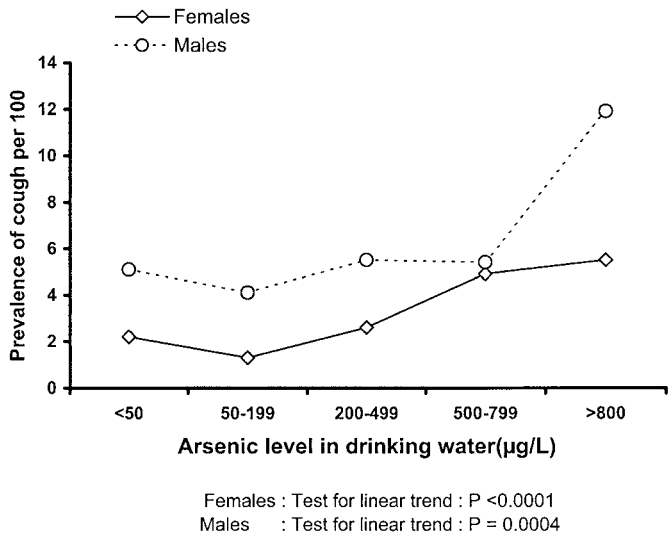

Fig. 2. Prevalence of cough per 100 in relation to arsenic exposure among nonsmokers.

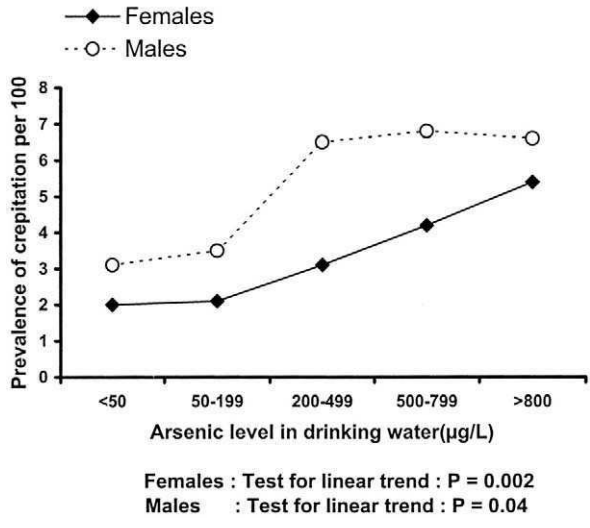

Fig. 3. Prevalence of crepitation per 100 in relation to arsenic exposure among nonsmokers.

The age-adjusted prevalence of weakness was also found to be increased with increasing As concentration in water in both sexes ($P < 0.0001$ in both sexes, Fig. 4).

Incidence of raised As concentrations in hair and nail in the chronically As-exposed people showing various clinical manifestation are given in Table 6. Overall incidence of elevated As concentrations in hair could be tested in 55 cases and in nail of 37 cases of chronic As toxicity with features of pigmentation with/without keratosis. Overall incidence of elevated As concentrations in hair and nail was found in 81.82 and 62% of cases, respectively.

TABLE 5

Age-adjusted prevalence odds ratio (POR) for respiratory effects in nonsmokers comparing those exposed to ≥500 μg/l with participants exposed to <50 μg/l (Guha Mazumder et al., 2000)

	Females		Males	
	Cases exposed to ≥500 μg/l	POR (95% CI)	Cases exposed to ≥500 μg/l	POR (95% CI)
All participants				
Cough	22	2.4 (1.4–4.1)	25	1.6 (1.0–2.7)
Crepitations	19	2.5 (1.4–4.4)	21	2.2 (1.3–4.1)
Shortness of breath	11	7.2 (2.8–18.5)	11	2.1 (0.9–4.4)
Weakness	47	7.2 (4.4–11.5)	20	6.9 (3.2–15.0)
With skin lesions				
Cough	7	7.8 (3.1–19.5)	14	5.0 (2.6–9.9)
Crepitations	8	9.6 (4.0–22.9)	12	6.9 (3.1–15.0)
Shortness of breath	4	23.2 (5.8–92.8)	5	3.7 (1.3–10.6)
Weakness	10	15.3 (6.5–35.8)	8	14.2 (5.2–38.7)
Without skin lesions				
Cough	15	1.8 (1.0–3.4)	11	0.9 (0.5–1.7)
Crepitations	11	1.6 (0.8–3.2)	9	1.2 (0.5–2.6)
Shortness of breath	7	5.2 (1.9–14.8)	6	1.5 (0.6–3.7)
Weakness	37	6.7 (4.1–11.1)	12	5.4 (2.3–12.8)

TABLE 6

Correlation of As level in hair and nail with skin manifestation of chronic As toxicity

	Arsenic level in hair			Arsenic level in nail		
	Total tested	>0.85 mg/kg	%	Total tested	>1.2 mg/kg	%
Pigmentation with/without keratosis	55	45	81.82	37	23	62

DISCUSSION

The clinical epidemiological survey demonstrated that pigmentation (8.82%) and keratosis (3.64%) were most specific diagnostic parameters of chronic As toxicity, as none of the people who drank water containing less than 0.01 As mg/l had these features. It is a matter of great concern that even drinking water with As concentrations between 0.01 and 0.05 could produce pigmentation and keratosis in some people who drank the water for more than 10 years. This further confirms the rationality of lowering the safe limit of As concentrations to 0.01 mg/l as recommended by WHO. Though liver enlargement was found in a higher number of cases (10.21%) in the As-exposed population its incidence in the unexposed population (As concentrations < 0.05 mg/l) was 2.9%. Thus though liver enlargement has a higher degree of sensitivity, it is less specific for the diagnosis of chronic arsenicosis. Similar was the observation in regard to lung involvement (11.68% vs 7.74%). Incidence of peripheral neuropathy was found in a fewer number of cases (4.7%), though it was significantly higher compared to control subjects ($P < 0.001$). There was no difference in the incidence of abdominal symptoms like pain in the As exposed and unexposed people and this symptom has the least probability of sensitivity and specificity in regard to the manifestation of chronic As toxicity.

It was earlier reported, on the basis of our hospital-based studies on 248 cases of severe chronic arsenicosis, that over and above pigmentation and keratosis the incidence of weak-

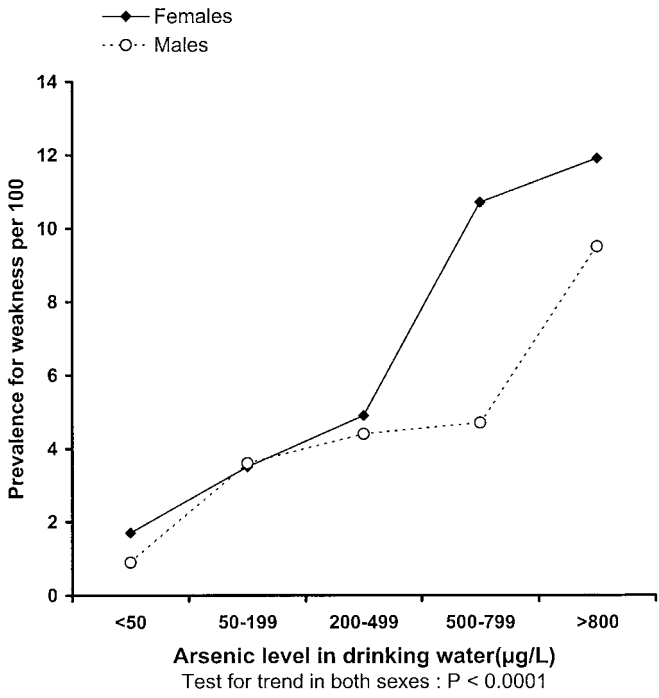

Fig. 4. Prevalence of weakness per 100 in relation to arsenic exposure among nonsmokers.

ness, hepatic involvement, chronic lung disease and chronic neuropathy were detected in a high number of cases (Guha Mazumder et al., 1999). The results of cross-sectional epidemiological study on 7683 people in West Bengal further substantiated that chronic As toxicity in man produces protean clinical manifestations.

The high incidence of hepatomegaly among an As-exposed population compared to control group appears to be As related as there was no preponderance of any of the etiological factors for hepatomegaly in the tropics (e.g., history of alcohol intake, jaundice, round worm infestation, HBV, HCV and amoebic seropositivity; Guha Mazumder et al., 1984) in any of the two groups compared. Further, the prevalence of hepatomegaly was found to have a linear relationship proportional to increasing exposure of As in drinking water in both sexes ($P < 0.001$; Fig. 1). Sporadic reports of liver involvement following intake of As in medicinal form have been published. A few reports mentioned the lesion in the liver to be non-cirrhotic portal hypertension. Two cases reported by Datta et al. (1979) and our data of 13 and 63 cases (Guha Mazumder et al., 1988; Santra et al., 1999) suggest that non-cirrhotic portal fibrosis occurs due to drinking As-contaminated water for a prolonged period. Of 15 cases of chronic arsenicosis described by Nevens et al. (1990), 11 cases were caused by intake of As in medicinal form. Most authors report the liver function profile to be normal or near normal in chronic arsenicosis with liver involvement. We reported earlier on a study of 93 cases with an elevated level of AST, ALT, ALP and Globulin in 25.7%, 61%, 27% and 20.7% of cases, respectively, of hepatomegaly due to drinking As-contaminated water (Santra et al., 1999). Liver function tests done on a few cases in our epidemiological study also substantiate our earlier observation.

This study further provides evidence that ingestion of inorganic arsenic in drinking water results in pulmonary effects manifested by cough, crepitations in the lungs and shortness of breath. With the exception of shortness of breath among females, the prevalence of

each outcome rose with increasing concentrations of arsenic in the primary drinking water sources (Figs. 2 and 3). The results in Table 5 indicate that in this population the presence of respiratory effects was largely confined to those who had arsenic-caused skin lesions. Why this should be so is not clear, but could be related to some underlying susceptibility to arsenic effect. In Chile, it was also noted that there were differences in respiratory disease in schoolchildren with skin lesions compared to those without the lesions (Borgono et al., 1977). In a study of 1774 cases of chronic arsenicosis patients living in 627 villages of the Inner Mongolia Autonomous Region of China drinking As-contaminated water (As concentrations 0.05–1.82 mg/l), 22.63% of cases showed respiratory system involvement (Guo et al., 1998).

Although information about the relationship between ingested arsenic and nonmalignant respiratory effects has so far only been reported from Chile and now India, and China, studies from arsenic-affected regions in Taiwan, Chile and Argentina show marked increases in lung cancer mortality (Chen et al., 1985; Wu et al., 1989; Chiou et al., 1995; Hopenhayn-Rich et al., 1998; Smith et al., 1998). It is of interest to note that many established lung carcinogens also cause nonmalignant lung disease including smoking, asbestos, and silica. The surprising characteristic of arsenic is that it seems to increase both malignant and nonmalignant respiratory disease following exposure.

Neuropathy is not always a constant feature of chronic arsenicosis. Though we have observed its incidence in 4.7% of people drinking As-contaminated water and similar effects are reported by Hindmarsh et al. (1977), Kiburn (1997), Ma et al. (1999) and Hotta (1989), such effects are not reported in studies from Argentina, Chile or Taiwan (WHO, 1981). Investigation of 530 individuals drinking As-contaminated ground water (0.95–2.3 mg/l) in large areas of Inner Mongolia, China, showed evidences of peripheral neuritis in 27% of cases and central nervous system dysfunction (abnormal EEF) in 40% of cases (Ma and Le, 1998).

Interestingly, weakness was found to increase dramatically with increase in As exposure through water in those with skin lesions (Table 5, PORs 15.3 and 14.2 for females and males, respectively). However, it was still markedly elevated in those with high current exposures who did not have skin lesions (age-adjusted PORs of 6.7 and 5.4). The reason why people exposed to high arsenic concentrations reported feeling weak is not clear. While arsenic can cause peripheral neuropathy, it is not known to cause central nervous system effects that could explain general feelings of weakness. None of our cases did have any evidence of peripheral vascular disease as has been reported in people in Taiwan and Chile (Borgono et al., 1977; Tseng et al., 1995). However, we noticed 3 cases of peripheral vascular disease among 248 cases of our hospital-based study where severe cases from the districts reported for their treatment (Guha Mazumder et al., 1998). It appears that the incidence of peripheral vascular disease in West Bengal is quite low.

It is interesting to note that some people presenting with features of chronic As toxicity, e.g., pigmentation with/without keratosis did not have elevation of As concentrations in their hairs and nails. Hence, estimation of As concentrations in hair and nail cannot solely be relied upon for the diagnosis of chronic arsenicosis.

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Possible Effects of Arsenic on Visual Perception and Visual–Motor Integration of Children in Thailand

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ABSTRACT

A zone of intelligence which reflects the neurological damage is perceptual organization and sensory–motor integration. To find out if children with high arsenic levels in hair had a deficit of intellectual ability, a cross-sectional study was conducted. The arsenic level in hair measured by AAS method was used as the independent variable. Neurological effects measured were visual perception and visual–motor integration (VMI) using the Motor-Free Visual Perception Test and a VMI test, respectively. Data were collected from 529 children aged 6–9 years in the Ronpiboon district between 16 January and 5 March, 1995. Controlling of potential confounders was conducted by restriction method and multiple classification analysis. Only 5% of the variance in visual perception of children was explained by arsenic significantly. We found a relationship between arsenic levels in hair and visual perception but a relationship with VMI was not present. In this study, arsenic did not affect the visual–motor integration of children. High arsenic levels in hair were associated with the impairment of visual perception. It is necessary to protect children from arsenic exposure. The health status of children with high arsenic levels should be systematically assessed.

Keywords: arsenic level in hair; MVPT; VMI; AAS; children

INTRODUCTION

The water supply for the population of Ronpiboon and nearby areas is from the Ronna River. It comes from the Ronna Mountains that are rich in tin, wolfram, and sulfidic ore deposits. The arsenic concentrations in these ores may range from traces to several percent. Other water sources are well water at 1–10 m depth (Division of Environmental Health, 1992; Choprapawon, 1994). The inhabitants in these areas are at risk of chronic arsenic exposure from drinking water with arsenic contamination. A study of chronic arsenism by stage of skin lesion found a high prevalence of severe skin lesions in the youngest age group (0–9 years) in 1987–1988 (Rodklai and Shindell, 1989). Some studies have reported that the high arsenic levels in hair which indicated chronic arsenic exposure were detected in children in the Ronpiboon subdistrict and some subdistricts in Ronpiboon district, Nakorn Si Thammarat province (Piamphongsant and Udomnitikul, 1989; Rodklai et al., 1994). Since 1987, there has been no monitoring system of drinking water quality. A recent survey of drinking water samples at Ronpiboon subdistrict still showed a high arsenic contamination ($\geq$0.01 ppm) 78.7% of total (Rodklai et al., 1994). Thus the children in this risk area are accumulating arsenic in their bodies through gradual chronic arsenic exposure. It is possible that the high arsenic level will affect the intellectual ability of those children.

Available evidence in experimental systems as well as in humans concerning effects of metallic compounds on the developing central nervous system, indicates that such effects should be considered further in relation to critical effects for arsenic, cadmium, lead, mercury and their compounds. Specifically for arsenic, developmental toxicity of possible significance in public health are neurological and neurobehavioral effects. At present, available evidence concerning the neurotoxic effects in children is considered by many as the critical effect, when exposures of the general population are considered. The fact that, in children, CNS effect, dose–response curves are less steep and thus less of the threshold type than in adults, is an important consideration (Nordberg, 1988).

Behavioral changes may be the earliest and only manifestation of neurotoxicity. Moreover, it is well known that extensive brain damage can occur with little or non-detectable clinical neurological deficit. Psychometric tests in toxicological and epidemiological research have been proposed to assess in an objective manner early manifestations of functional neurological deficit and to detect preclinical central nervous system changes that may be due to environmental neurotoxic agents (Valciukas and Lilis, 1980). In selecting neurobehavioral tests, the tests should be non-language-dependent and culturally fair. In field studies, these tests have to be simple, inexpensive, and easy to carry out; the administration and the scoring system have to be objective and based on the evaluation of the subject's performance. Performance tests seem to be desirable in these circumstances (Valciukas and Lilis, 1980).

Neurological defects are found in three broadly delineated zones of intellectual skill: (1) perceptual organization and sensory–motor integration; (2) logic and thought; and (3) language (Pope and Scott, 1967). In our study, we selected the Motor-Free Visual Perception Test (MVPT), and the Visual–Motor Integration Test (VMI Test) to determine the outcome for visual perception score, and VMI score of children. The study aimed to find the relationships of arsenic concentrations in hair with visual perception, and visual–motor integration of children.

METHODOLOGY

Study Population

Subjects were children aged 6–9 years who were born in the subdistricts Ronpiboon and Soa Thong between 1986 and 1989. They must have lived in those areas since birth and

their parents must be a married couple, live together and look after them. There were 838 children who met these criteria. We selected 529 from the 838 subjects (63.1%) with simple random sampling. The sample size was calculated by the package program EPI-INFO version 5. It was based on a 95% level of confidence, 90% power of the test, a ratio of unexposed to exposed of 1 : 2 and a 1.79 relative risk of visual perception abnormality.

Data Collection

We collected arsenic concentration data in hair reflecting chronic arsenic exposure as the independent variable by cutting 1 g of each child's hair. These hair samples were sent to the Faculty of Pharmaceutical Science, Prince of Songkla University, for arsenic analysis. Visual perception and VMI score were dependent variables which were obtained by using MVPT and VMI tests. Potential confounders such as gender, birth order, birth weight, prenatal factors, perinatal factors, postnatal factors, breast feeding, illness history, food intake, family size, family income, parents' quarrel, child rearing, the mother's education and occupation, the father's education and occupation, history of the father's slow learning were collected from the child interview form, the mother's interview form and the father's questionnaire. We measured vision and hearing of the children with Snellen's chart and Impact Audiometer Model 1001. Maternal intelligence was assessed with the Standard Progressive Matrices slides. The period of data collection was between 15 January and 5 March, 1995.

Statistical Analysis

Distribution of arsenic concentrations in hair, visual perception score and VMI score were presented in frequencies and percents. Homogeneity of some variables was tested by the chi-square test and Student's *t*-test. To explore the relationships between arsenic concentrations in hair and outcome variables we used the multiple classification analysis. These methods were analyzed by the statistical package of SPSS/PC+ version 4 (Norusis, 1990).

RESULTS

Arsenic Level in Hair

The arsenic level in hair was analyzed by atomic absorption spectrophotometry using a GBC 906 automatic multi-element atomic absorption spectrophotometer with the GBC HG 3000 hydride generator (Chapple and Danby, 1990). The arsenic levels ranged from 0.48 to 26.94 μg/g. The mean arsenic concentration was 3.52 μg/g (SD = 3.58), and the median arsenic concentration was 2.42 μg/g. Only 44 of 529 (8.3%) samples had normal arsenic concentrations ($\leq$1 μg/g). Half of them had arsenic concentrations between 1.01 and 3 μg/g (Fig. 1).

Poor Visual Perception and Visual–Motor Integration

The visual perception score of each child was compared with the score of children in the Hintok subdistrict of the same age (Siripitayakunkit and Thonghong, 1997). The cut off point for poor perception was the mean minus one SD in each age level. The study found that 21% of the children had poor visual perception as shown in Fig. 2. The VMI standard score has a mean of 100 and a standard deviation of 15 for each age group (Berry, 1987). A poor VMI subject was the child who had a standard score below 85. We found that poor visual–motor integration was 17.6%.

Association between Arsenic Level and Visual Perception

Almost 40% of the perception scores was explained by arsenic, father's occupation and mother's intelligence score after adjusting for independents and one covariate (age). This

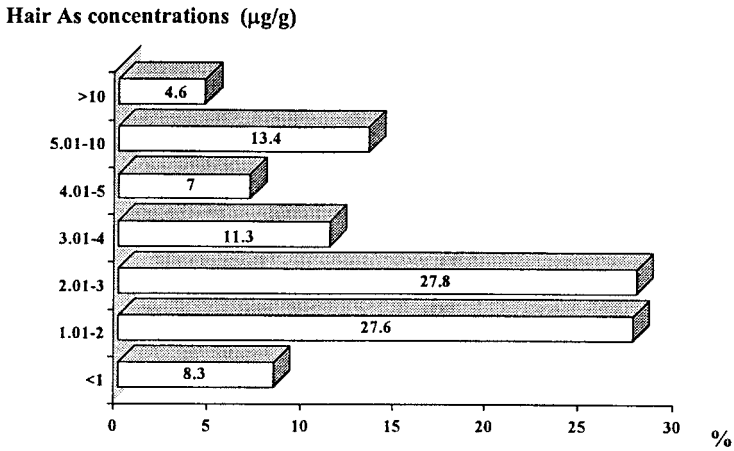

Fig. 1. Percentage of arsenic concentrations in hair of children aged 6–9 years, Ronpiboon district, Nakorn Si Thammarat province, 1995.

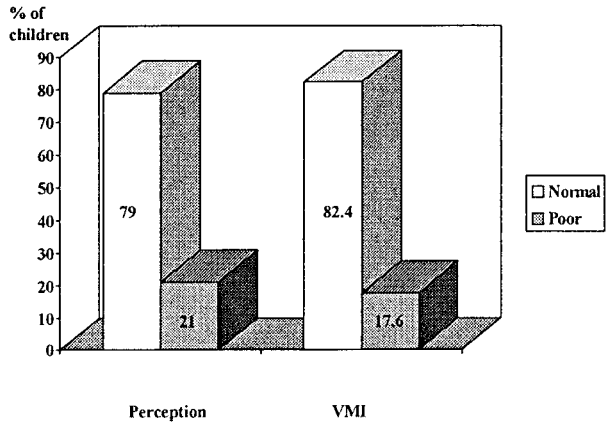

Fig. 2. Percentage of poor visual perception and poor VMI of children, Ronpiboon district, Nakorn Si Thammarat province, 1995.

set of variables explained the variance in the visual perception scores significantly ($P < 0.001$) as shown in Table 1.

After controlling for confounders, only 5% of the variance in visual perception was explained by arsenic ($P = 0.01$). This explains why the mean perception score of the low arsenic group was 0.35 points higher, the mean perception score of the high arsenic group was 0.06 points lower and the mean perception score of the very high arsenic group was 0.54 points below the grand mean perception score (20.57). The mean perception score for each arsenic group is presented in Table 2.

Association between Arsenic Level and Visual–Motor Integration

The results showed that arsenic could not explain the variation in the VMI score. Three variables, father's education, mother's occupation and mother's intelligence score significantly affected the variance of the VMI score ($P < 0.05$). Nevertheless, only 11% of the VMI score could be explained by arsenic level and those variables.

TABLE 1

Multiple classification analysis of perception score in children aged 6–9 years, Ronpiboon district, 1995 (grand mean = 20.57)

	n	Unadjusted		Adjusted for independents + covariates		*P*-value
		SD	Eta	SD	Beta	
Arsenic (μg/g)			0.10		0.05	0.01**
Low (≤2)	190	0.64		0.35		
High (2.01–5)	244	−0.09		−0.06		
Very high (>5)	95	−1.07		−0.54		
Father's occupation			0.17		0.12	0.00***
Farmer	211	−0.82		−0.52		
Labourer and miner	248	0.03		0.05		
Trader and Government employee	70	2.36		1.73		
Maternal intelligence score			0.14		0.13	0.01**
Very low (≤10)	39	−0.55		−0.49		
Low (11–20)	255	−0.52		−0.61		
Medium (21–30)	137	−0.07			0.28	
High (>30)	98	1.67			1.40	
Multiple R^2					0.39	
Multiple R					0.62	

Level of significance: ** $P < 0.01$, *** $P < 0.001$.

TABLE 2

Children's mean perception score in different arsenic groups after controlling for confounders

Arsenic groups (μg/g)	Mean (score)	95% CI (score)
Low (≤2)	20.92	20.52–21.32
High (2.01–5)	20.51	20.11–20.91
Very high (>5)	20.03	19.63–20.43

Father's education accounted for 18% of the variance in VMI score after controlling for confounders ($P < 0.01$). Children of fathers in the low education group (≤primary level) had mean VMI scores 1.24 points less than the grand mean (95.94). Children of fathers in the high education group (>high school level) had mean VMI scores 5.74 points higher than the grand mean. Mother's occupation accounts for 15% of the variance in VMI score after controlling for confounders ($P = 0.01$). Children of mothers who were farmers had mean VMI scores 1.29 points lower than the grand mean. Children of mothers who were in the commercial and government employee group had mean VMI scores 3.74 points higher than the grand mean (Table 3). Intelligence of the mother accounted for 13% of the variance in VMI score after controlling for confounders ($P = 0.05$). The children in the group with very low maternal intelligence had mean VMI scores 1.85 points lower than the grand mean, but those in the group with high maternal intelligence had mean VMI scores 3.23 points higher than the grand mean.

DISCUSSION AND CONCLUSIONS

Poor visual perception was found in 21% of all children. This finding was higher than the level of poor visual perception found in children in the Hintok subdistrict. The reliability

TABLE 3

Multiple classification analysis of VMI score in children aged 6–9 years, Ronpiboon district, 1995 (grand mean 95.94)

Variables	*n*	Unadjusted		Adjusted for independents + covariates		*P*-value
		SD	Eta	SD	Beta	
Arsenic (μg/g)			0.03		0.03	0.82
Low (≤2)	190	−0.42		−0.50		
High (2.01–5)	244	0.11		0.21		
Very high (>5)	95	0.57		0.46		
Father's education			0.25		0.18	0.00***
≤ primary school	382	−1.71		−1.24		
high school	100	2.54		2.04		
> high school	47	8.52		5.74		
Mother's occupation			0.20		0.15	0.01**
Farmer	233	−1.96		−1.29		
Labourer	116	−0.85		−0.90		
Housewife	95	1.35		0.93		
Trader and government employee	85	5.03		3.74		
Maternal intelligence score			0.22		0.13	0.05*
Very low (≤10)	39	−2.15		−1.85		
Low (11–20)	255	−1.58		−0.85		
Medium (21–30)	137	−0.22		−0.21		
High (>30)	98	5.42		3.23		
Multiple R^2				0.11		
Multiple R				0.33		

Level of significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

study of the MVPT found that 16.5% of 158 children aged 6–9 years had poor visual perception (Siripitayakunkit and Thonghong, 1997). The percentage of poor visual perception was only minimally related to the arsenic level, explaining only 5% of the variance in visual perception after controlling for confounders. The study found poor VMI in 17.6% of all subjects. The 1989 VMI norms based upon findings from 2674 American children aged 6–9 years (Berry, 1989) were applied to calculate the percentage with a poor VMI score (below a standard score of 85). The proportion of children with poor VMI scores was approximately 16% (15.8%) of the total. This study revealed a slightly higher percentage with poor VMI scores than established in the VMI norms. The percentage of children with poor VMI scores was not associated with arsenic concentrations in hair. Results showed no significant association between arsenic level and VMI score after controlling for confounders.

The Motor-Free Visual Perception Test (MVPT) and Visual Motor Integration Test (VMI Test) were used in this study to determine the intellectual ability of children. Neither perception score nor VMI score differed significantly with varying arsenic concentrations when they were analyzed by the *t*-test. However, after adjusting for independent variables and covariate, there was a significant difference in visual perception scores ($P = 0.01$), although only 5% of the variance in visual perception could be explained by arsenic level. Three factors that affected visual perception significantly were the child's age, the intelligence score of the mother, and the occupation of the father. The present study found a significant inverse relationship between arsenic concentrations in hair and the visual perception score, though it was not a strong relationship.

After controlling for confounders by multivariate analysis, the result still showed no relationship between arsenic level in hair and the VMI score. We concluded that arsenic did

not affect the visual–motor integration of children. The factors that significantly affected children's visual–motor integration were education of father, occupation of mother and maternal intelligence ($P \leq 0.05$). The findings revealed that children with high arsenic accumulation from chronic arsenic exposure were impaired in visual perception skills. They had no motor disability as shown by the similar VMI scores of all children.

The brain growth spurt runs from mid-pregnancy to about 3 to 4 years in humans. This period is the focus of early central nervous system (CNS) development and is characterized by glial proliferation, and subsequent myelination. Exposure to adverse environmental circumstances and abnormal hormonal influences are dangerous at this time. They can lead to permanent impairment or alteration of CNS functions that cannot be reversed by later attempts at correction (Meyer-Bahlberg et al., 1978; Brook, 1982). In humans, effects of metallic compounds on the developing CNS indicate that arsenic can have a critical effect (Nordberg, 1988). Children living in an arsenic contamination area are continuously exposed to low-dose arsenic since birth. The arsenic accumulation can be detected in hair which reflects chronic arsenic exposure. Later development problems among children who had high arsenic concentrations in their hair suggest that chronic low-dose arsenic ingestion might affect CNS.

Since there was no monitoring of arsenic levels using biological sampling since birth, and no prospective growth and intelligence records for children in the Ronpiboon district, a prospective study of chronic arsenic effects could not be performed in a limited time. To clarify any association, further research should be conducted in a follow-up study. However, the findings of an inverse association between arsenic concentrations in hair and visual perception of children living in the Ronpiboon district are sensitive and of public concern. Rapid and proper management from responsibility organizations is needed.

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Interactions between Arsenic and Other Factors in Relation to Carcinogenicity

Irva Hertz-Picciotto

ABSTRACT

The possibility that there are interactions between arsenic and other factors in terms of the impact on cancer risk is important for any evaluation of possible regulatory measures related to this pollutant in air or water. If the effects of arsenic are stronger among certain populations because of specific lifestyle or genetic factors, then risks must be assessed according to the distribution of those factors in the population of concern. This paper reviews the evidence that arsenic interacts with other factors in increasing the risk for cancer among exposed populations. First, I present the relevant definitions; then I review examples from the literature relating lung cancer to occupational arsenic exposure and interactions with smoking; finally, I describe published data on carcinogenicity of ingested arsenic according to potential effect modifiers such as nutritional status, age, gender, genes, smoking, and infections.

Keywords: arsenic; interaction; synergism; nutrition; genetics; smoking; infection; liver function

INTRODUCTION

Any assessment of risk from an environmental pollutant must consider the possibility that the exposure of interest differentially affects certain subsets of the population. If arsenic is found to interact with other factors, be they exogenous or endogenous, the distribution of these factors becomes of importance to regulators. The public health impact of any particular regulatory decision is directly related to the strength of the interaction, the magnitude of the arsenic exposure, and the prevalence of the modifying factors. Thus, the question of whether there are interactions between arsenic and other factors in terms of the impact on cancer risk is important for any evaluation of public health concerns and possible regulatory measures related to this pollutant in air or water. If the effects of arsenic are stronger among certain populations because of specific lifestyle or genetic factors, then risks need to be assessed according to the distribution of those factors in the population of concern.

DEFINITIONS

A discussion of interactions between arsenic and other factors must begin with definitions. First, *interaction* is defined to occur when the measure of association between an exposure (here, arsenic) differs according to the level of another factor. The presence of this difference is also referred to as 'heterogeneity', or 'effect modification', or sometimes 'effect measure modification'. The other factor might be age, gender, smoking status, GST-T1 polymorphism, use of medications, nutritional status, etc. It could be a risk factor for the disease in question (here, cancer) or it may not act alone at all, but instead simply intensify the impact of another risk factor.

Consider the interaction as shown in Tables 1 and 2, where there are two possible exposures: A (arsenic) and/or X (some other risk factor). The notation is as follows:

r_{ax} = rate of disease in those with neither A nor X present
r_{Ax} = rate of disease in those with A present but not X
r_{aX} = rate of disease in those with X present but not A
r_{AX} = rate of disease in those with both A and X present

where rates of disease are defined as the number of cases divided by the number of person-years of observation in the population from which the cases arose.

Table 1 shows these rates; in Table 2, each rate has been divided by what might be

TABLE 1

Rates of disease according to exposures to A or X

	A absent (unexposed to arsenic)	A present (exposed to arsenic)
X absent	r_{ax}	r_{Ax}
X present	r_{aX}	r_{AX}

TABLE 2

Rate ratios of disease by status of exposure to A or X, as compared with those unexposed to both

	A absent (unexposed to arsenic)	A present (exposed to arsenic)
X absent	$r_{ax}/r_{ax} = 1$	r_{Ax}/r_{ax}
X present	r_{aX}/r_{ax}	r_{AX}/r_{ax}

referred to as the background or baseline rate of disease, i.e., r_{ax}, in order to obtain rate ratios. The rate ratio is a frequently used measure of association. However, other measures of association are also possible, such as the rate difference, the risk difference, etc. Since it is possible for the rate ratios at two levels of the second factor to be the same but the rate differences to be heterogeneous, one can only speak of interaction in reference to a specific measure of association or scale (Hertz-Picciotto et al., 1992). In other words, interaction on an additive scale does not necessarily imply interaction on a multiplicative scale, and vice versa.

A second set of definitions relates specifically to the additive scale. Synergism occurs when the additive measure of association in the presence of both factors is greater than the sum of the measures for either factor alone. Antagonism occurs when the measure of association in the presence of both factors is smaller than the sum of the measures for either factor alone (Hertz-Picciotto et al., 1992).

CARCINOGENIC INTERACTIONS FOR SMOKING AND OCCUPATIONAL (INHALED) ARSENIC

Table 3 provides three examples of carcinogenicity from arsenic and smoking (Pershagen et al., 1981; Enterline, 1983; Pershagen, 1985). The first shows that the relative risk for lung cancer, comparing retired arsenic smelter workers to the male population in the state where the study was conducted, among nonsmokers is 5.1. If we compare smokers to nonsmokers among the general population of males, the relative risk is 7.2. If the effects were multiplicative then we would expect the arsenic-exposed workers who were smokers to have a relative risk of about 35. The actual relative risk is 20.7, indicating that there is interaction on the multiplicative scale: the effects of smoking and arsenic exposure are less than multiplicative. If we express these numbers on the additive scale, we obtain an excess relative risk (ERR) for arsenic exposure among nonsmokers of 4.1, an ERR for smoking in the general population of males of 6.2, and an expected ERR, under an additive model, of 11.3 (= background + arsenic ERR + smoking ERR). The actual ERR is 19.7, indicating a synergistic effect.

The second example in Table 3 also shows a synergistic effect, but in this Swedish study, the effects of smoking and arsenic exposure are multiplicative (predicted relative risk = $3 \times 4.9 = 14.7$; observed relative risk = 14.6). The third example, also from Sweden, suggests that residential exposure to arsenic (i.e., exposure among persons living near a smelter) has a multiplicative effect with smoking on lung cancer, whereas occupational exposure shows a less than multiplicative relationship with smoking vis-à-vis the induction of cancer. Both

TABLE 3

Comparison of relative risks for lung cancer according to arsenic exposure and smoking status

		No As	As-exposed by employment in smelter
Enterline et al., 1983:	Nonsmokers	1.0	5.1
	Smokers	7.2	20.7
Pershagen et al., 1981:	Nonsmokers	1.0	3.0
	Smokers	4.9	14.6
Pershagen, 1985:	Nonsmokers	1.0	8.4
	Smokers	8.3	26.2
			As-exposed through residence near a smelter
	Nonsmokers	1.0	2.3
	Smokers	8.3	17.5

types of exposure seem to indicate synergism. We have found that the body of literature on occupational arsenic exposure and smoking clearly supports a synergistic effect of these two exposures (Hertz-Picciotto et al., 1992).

CARCINOGENIC INTERACTIONS FOR INGESTED ARSENIC

Numerous factors have been or might be hypothesized to interact with arsenic ingested from drinking water in increasing the risk of, or altering susceptibility to, cancer. Table 4 summarizes these. Below, we review evidence for such effect modifiers.

Genetic Susceptibility: Methylation Capacity

Methylation capacity has been hypothesized to play a role in susceptibility to arsenic-induced carcinogenicity. Hsueh et al. (1997) classified subjects from a Taiwanese study according to the percent of arsenic excreted as monomethyl arsonic acid (MMA) in urine and also by cumulative arsenic exposure. These authors found that the multivariate adjusted odds ratios for skin cancer differed in these four groups. Within the low cumulative arsenic exposure group, those with a higher percentage of urinary MMA showed a relative risk of 3.0 compared to those with a lower percentage (<26.7%). Among those with a low excretion of MMA, a higher cumulative arsenic exposure was associated with an 8-fold increased risk of skin cancer. However, when comparing the highly exposed, high MMA excreters to those with low exposure and low MMA excretion, the OR was 24. These findings suggest that methylation capacity could substantially alter the additive impact of arsenic exposure, i.e., the number of excess cases in a population. Since arsenic exposure was simply dichotomized into low and high cumulative exposures, and since these were fairly high levels, studies are needed with a finer stratification of arsenic exposure and perhaps of methylation capacity. Further data are needed to determine whether these interactive effects are linear or not.

Genetic make-up of a population or individual is likely to modify the carcinogenic effect of arsenic exposure, and methylation capacity is one primary suspect for genetic control. Chiou et al. (1997) present data from Taiwan suggesting that both GST-T1 and GST-M1 may alter methylation capacity. Other data related to possible genetic factors are reported by Hopenhayn-Rich et al. (1996). In a study of Chileans in the Atacama Desert, those of European descent had an increased ratio of excreted MMA : DMA as compared with native populations (Atacameños) who have lived in the local area for centuries.

Nutritional Factors

Hsueh et al. (1997) report on nutritional status of their study population. They found that in a three-year follow-up, participants with a higher intake of beta-carotene had lower risk

TABLE 4

Factors that are potential effect modifiers of the relationship between arsenic and cancer

Endogenous	Genetic factors (methylation capacity)	GST-M1 polymorphism GST-T1 polymorphism
	Gender	
	Morbidity	Hepatitis B surface antigen, liver disease
	Physiology	Liver function
	Age	
Exogenous	Nutrition	Beta-carotene
	Smoking	
	Medications	

for skin cancer in this arsenic endemic area. However, it is not clear whether beta-carotene actually altered the effect within strata of arsenic exposure.

Experimental data also point to the possible importance of nutritional status. Vahter and Mirafante (1987) administered diets deficient in choline, methionine or protein to rodents. Their findings indicate that, as compared with controls, the animals given any one of the three deficient diets demonstrated reduced excretion of total arsenic and of dimethyl arsinic acid (DMA) and reduced levels of S-adenosyl-methionine in the liver. Those with the low methionine diet excreted more inorganic arsenic. In human data, differences have been noted in breast milk choline concentrations when comparing women from Ecuador to those in Boston (Zeisel et al., 1982), but the consequences of this finding are unknown.

Smoking

Although there is an abundant literature on interactions between inhalation exposures and smoking, much less has been reported in relation to ingestion of arsenic. Moore et al. (1997) showed that, in a population in Chile with high arsenic exposure, exfoliated bladder cells were more likely to be micronucleated in smokers than in nonsmokers; these authors also found that the provision of drinking water with low arsenic resulted in a greater decline in bladder cell micronuclei among smokers as compared with nonsmokers. Thus, after consumption of drinking water with reduced arsenic, smokers and nonsmokers had similar concentrations of micronucleated bladder cells in urine, suggesting stronger genotoxicity of ingested arsenic among smokers than nonsmokers.

Hopenhayn-Rich et al. (1996), also in a study conducted in Chile, compared MMA : DMA ratios in smokers and nonsmokers. The ratio was 0.26 in smokers and 0.18 in nonsmokers ($P < 0.001$). Thus, nonsmokers appear to be able to carry out the second methylation step more readily than smokers.

Gender

Chen et al. (1985, 1988) and Steinmaus et al. (2000) report a much higher relative risk for bladder cancer among females as compared with males in arsenic endemic areas of Taiwan. However, among those unexposed to arsenic in drinking water, the bladder cancer rates among females were much lower than those among males. In fact, among arsenic-exposed persons, the rate for bladder cancer was similar for men as compared with women. It appears that there is a higher background rate among men because of other exposures, better ascertainment, or genetic predisposition. Thus, it is unclear whether the differing gender-specific risk ratios are important per se, or merely reflect, for instance, possible differences in smoking habits.

A few other studies have examined mechanisms potentially related to gender–arsenic interactions. In Chile, men showed higher MMA : DMA ratios (0.23 vs. 0.17) (Hopenhayn-Rich et al., 1996). Gosenbatt et al. (1994), using an in vitro assay, showed that lymphocyte proliferation is impaired by arsenic and that the impact in females is greater than in males.

Age

It might be hypothesized that the carcinogenicity of arsenic could be age-dependent. Few studies have reported age-stratified data. Prevalence ratios from Tseng et al. (1968) indicate strong synergism with age in the induction of skin cancer by arsenic from drinking water in Taiwan, i.e., far greater than additive effects. There appeared to be a modest suggestion of decreasing prevalence ratios as the population aged, comparing the high vs. low exposure groups (i.e., less than multiplicative effects). Nevertheless, while excesses were seen at all ages in both sexes, most cases induced by arsenic were in persons over 60 years of age.

Infections and Liver Function

The liver is the site of methylation, and hence any impairment of liver function might be hypothesized to influence arsenic toxicity or carcinogenicity. Hsueh et al. (1995) showed that within an arsenic-exposed population, those persons displaying hepatitis surface B antigen and with measured liver impairment were more likely to develop skin cancer than those displaying the antigen but with no measurable liver impairment. A reduced MMA : DMA ratio in urine is also observed in association with liver disease (Buchet et al., 1984).

DISCUSSION AND CONCLUSIONS

While it is known that for those exposed to arsenic occupationally through inhalation, smoking increases the arsenic-associated risk for developing lung cancer, it is unclear whether smoking has a similar modifying effect in relation to carcinogenicity of ingested arsenic. Several studies do suggest that smoking influences methylation, but findings require confirmation in different populations, and the impact on cancer risk and attributable cases is unclear. Similarly, genetic factors also appear to influence methylation capacity, but the data are sparse. Liver disease or infections that alter liver function are also suspect modifying factors. Attention should be paid to those who are taking medications that may compromise liver function.

Nutritional factors may also play a role in enhancing or mediating arsenic carcinogenicity, but at this point, there are no direct data on humans supporting this hypothesis. In spite of clear experimental data, it is not clear whether a nutritional deficiency in choline or methionine could measurably alter methylation capacity in humans, and if so, how deficient the diet would have to be.

At this point, data are insufficient regarding male/female differences in susceptibility. Previously reported disparities in arsenic-related relative risks for bladder cancer were due to an exceptionally low risk in nonexposed females.

While regulators should be aware of the potential modifying influences of smoking, medications, diseases, nutrition, and genetic factors, quantitative data are currently lacking. Given the many uncertainties, further research relating ingested arsenic to cancer is needed in a wide range of populations whose concomitant exposures vary. Stratification of analyses relating arsenic to cancer risk on these potential susceptibility-altering factors may highlight those who belong to vulnerable subgroups in various populations. These data can then lead to more informed policy decisions.

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Arsenic in Drinking Water from Groundwater in Transylvania, Romania: an Overview

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ABSTRACT

In the northwest region of Transylvania (Bihor and Arad counties), Romania, drinking water contains arsenic as a result of geochemical characteristic of the land. The present paper summarises the knowledge of the studies carried out in the region between 1992–1995. The geographical distribution of arsenic in drinking water in counties Bihor and Arad is spotty, with a mixture of high (mostly in the rural area) and low arsenic concentrations in contiguous areas (0–176 μg As/l). Estimates indicated that about 36,000 people are exposed to arsenic via drinking water in concentrations ranging from 11 mg/l to 48 mg/l, and about 14,000 inhabitants are exposed to water arsenic levels exceeding 50 mg/l. Significant and important differences related to the incidence and/or prevalence of cancers, cutaneous disorders, peripheral neuropathy cardiovascular diseases and also spontaneous abortions were observed in the exposed population versus the unexposed population. As a result of urinary examinations, blood pressure and haematocrit measurements in the two groups a significant difference was noticed for hematuria and albuminuria, hypertension and low haematocrit. The findings of the surveys in the northwest region of Transylvania on arsenic emphasize the need for and the importance of the extension of exposure assessment and evaluation of health impact to intermediary concentrations of arsenic in drinking water.

Keywords: arsenic; exposure; drinking water; health effects

In Romania, several areas have been affected by natural arsenic contamination, others areas being contaminated from anthropogenic sources (non-ferrous industry). In the north-west region of Transylvania (Bihor and Arad counties), drinking water contains arsenic as a result of geochemical characteristic of the land, as well as in Bekes district located southeast of Hungary, causing a non-occupational exposure. The populations in this cross-border region obtain their drinking water from the naturally contaminated sources. This region is known as having elevated arsenic levels of water dating back from the 1940s. The main purposes of the studies carried out in the region were to investigate the health impact of arsenic-contaminated drinking water, assess individual variation in susceptibility, and improve the scientific basis of arsenic risk assessment. The specific objectives were: (1) to describe the current patterns of exposure of the general population to arsenic through ingestion of drinking water; (2) to describe the inter-individual variation in arsenic methylation and the effect of age, gender, dose of arsenic; (3) to reconstruct for the study population, historical arsenic intakes from drinking water sources; (4) to develop in areas with continued elevated arsenic in drinking water, a risk management strategy for achieving safe drinking water.

The estimated incidence of morbidity reported by G.P.'s for a period of 11 years (1981–1991) pointed out important differences between the exposed and the unexposed population. A higher incidence rate for bladder cancer was observed among the adults living in the exposed area (above 100 μg/l arsenic in underground drinking water) versus the control areas (<50 μg/l arsenic in drinking water). Lung, skin, kidney, leukaemia and other lymphatic and haematopoietic cancers were not elevated in the exposed population. The estimated incidence of anaemia, high blood pressure, rhythm disorders and peripheral neuropathy were higher in the exposed localities. Also there was a significant difference regarding the incidence of registered spontaneous abortions between the exposed and the control areas. A significant increase in the incidence of peripheral vascular disorders and cirrhosis was not found between the settlements exposed to high levels of arsenic in drinking water as against the control (Dora, 1995; Gurzau and Lazar, 1995; Surdu et al., 1997).

The results of a cross-sectional study, conducted in the same region in 1992 (Gurzau and Lazar, 1995), using a questionnaire, surveyed one exposed community and one unexposed community. The survey showed an increase prevalence of reported morbidity for cutaneous disorders (hyperpigmentosis, hyperkeratosis, epithelioma), peripheral neuropathy (parestesis in the extremities) and also cardiovascular diseases (hypertension, myocardial infarction, strokes) in the exposed group versus the control. As a result of urinary examinations, blood pressure and haematocrit measurements in the two groups a significant difference was noticed for haematuria and albuminuria, hypertension and low haematocrit. The lack of information on confounding factors, the small number of subjects and the limitations of the cross-sectional study design could be some of the reasons why we did not notice a relationship between arsenic exposure in drinking water and peripheral vascular disorders or skin, lung, and kidney cancers, that were reported in several studies.

A cross-sectional study to assess the arsenic exposure of human populations via drinking water was performed in the west part of the counties Bihor and Arad in 1995 (Gurzau and Lazar, 1995). The region taken under study had a population of about 600,000 people, of whom 77% in the urban area and the rest in the rural area. The arsenic levels were measured by the method of atomic absorption spectrophotometry in 134 water samples, collected from six towns and 80 settlements. Also were collected data concerning depth of water supply sources and the time period that has passed since that they were opened. Investigation of sources identified private and public wells with 10 to 632 m depth, and a public piped water system with a surface source (river), with the period of use ranging between one and more than 100 years.

Higher arsenic content was found in older underground wells. The range of arsenic concentrations was from 0 to 176 μg/l, 12.8% of the settlements had levels above the current national and WHO standard of 50 μg/l. A percentage of 37.2 of the settlements exceeded 10 μg As/l, maximum limit proposed in the provisional guideline values by WHO and US EPA. The wells with higher arsenic concentrations are mostly located in rural areas (Gurzau and Lazar, 1995; Surdu et al., 1997).

Estimates indicated that about 36,000 people are exposed to arsenic via drinking water in concentrations ranging from 11 μg/l to 48 μg/l, and about 14,000 inhabitants are exposed to arsenic levels in exceeding 50 μg/l. No evidence was found regarding contamination with arsenic of water supplies of the main cities (Gurzau and Lazar, 1995; Surdu et al., 1997).

The geographical distribution of arsenic in drinking water is spotty, with a mixture of high and low arsenic concentrations in contiguous areas, but the settlement areas with arsenic content in drinking water above 25 μg/l and with an estimated population exposed over 15%, are located especially in the southwest part of Bihor county and in the northwest part of Arad county (Gurzau and Lazar, 1995; Surdu et al., 1997).

The findings of the surveys in the northwest region of Transylvania as well as the scientific uncertainty regarding the human health effects of arsenic in intermediary concentrations (between 10 and 50 mg As/l) emphasize the need for and the importance of the extension of exposure assessment and evaluation of health impact to such levels of arsenic in drinking water.

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Preliminary Analysis of Lung Cancer Incidence in Arsenic Exposed Population

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ABSTRACT

The subject of our analysis was a database of 844 lung cancer cases collected within 15 years (three 5-year intervals) in a region polluted by emissions from burning of coal with high arsenic content ranging between 900 and 1500 g per metric ton of dry coal. The standardized incidence of lung cancer (each confirmed by biopsy or autopsy histological examination) in a district with population ~125,000 in non-occupational settings ranged from 67.57 to 88.63 in men and from 7.04 to 10.88 in women per 100,000 (study base 961,960 man/year and 977,940 woman/year) while relevant data for occupational settings (male workers of power-plant burning arsenic-rich coal) ranged from 31.76 to 988.94 per 100,000 (study base 21,360 man/year). Smoking habit was carefully registered in all cancer patients including lung cancer cases and the potential contribution of both factors is subject of a current analysis. Exposure assessment was based on biological monitoring. Determination of arsenic was done in groups of 10-year-old boys (in non-occupational settings) by analyzing hair and urine samples at different localities situated up to distances of 30 km from the local power plant. The results obtained seemed to suggest that arsenic is probably a promotor rather than a true carcinogen, similarly to a previous case of our analysis of arsenic-exposure-related skin basalioma incidence. The non-threshold concept of arsenic carcinogenicity seems not to be supported by the results of our preliminary meta-analysis.

Keywords: cancer epidemiology; biological monitoring; arsenic toxicity; lung cancer incidence

INTRODUCTION

The trace element content of coal is known to vary with the specific geological conditions of mines (Niu et al., 1997; Thornton and Farago, 1997). Ecological aspects of the excessive contamination of the environment by arsenic due to burning coal of high arsenic content including the extinction of honeybee colonies up to 30 km in the direction of the prevailing winds from the power plant, and a harmful influence of arsenic on the reproductive functions of domestic animals have been reviewed recently (Bencko, 2000). Neurotoxicity and immunotoxicity phenomena encountered in humans exposed to arsenic in environmental (Bencko et al., 1977) and occupational settings (Buchancová et al., 1998) have been described as well.

Assuming that the arsenic exposure leads to the development of malignant tumors, at a high exposure rate, we conducted a retrospective epidemiological study, the objective of which was to verify the anticipated increased rate of tumor mortality among employees of the power-plant under study. The mortality pattern was analyzed among workers of a power-plant, combusting coal with a high level of arsenic and compared with the mortality structure of deceased employees from three other coal-fired power plants, where the arsenic content in coal was 'normal'. To ensure homogeneity of the investigated groups, this study involved only the male employees of the above power-plants (female employees comprised about 20% of all workers).

The ascertained numbers of man/year employees during the whole period of study (1960–1978) in power-plants burning arsenic-rich coal were 15,768 and 17,363 controls.

The rate of tumor mortality among the exposed subjects who died before age 60 was 38% (in the control group 23%), among those who died after 60 it was 51% (in the controls 43%). This increase in the tumor mortality rates was in spite of being evident not statistically significant due to the small numbers involved.

Whereas the youngest case of tumor-caused death in the control group was 45 years old, in the exposed group the youngest case was 32 and in the age category below 45 years there occurred 26.9% of a total of tumor-caused deaths in the exposed group. These differences are statistically significant. The analysis shows that the mean age of cancer mortality in the exposed group is 5 years lower: 55.9 years versus 61.2 years of the control group ($P > 0.05$) (Bencko et al., 1980). The basic disadvantage of the retrospective study was the difficulty in controlling for other risk factors, such as tobacco smoking. To overcome the disadvantage of a retrospective study, we designed a population-based prospective study, beginning in the mid-1970s (Bencko and Götzl, 1994).

MATERIAL AND METHODS

This study is a continuation of our previous report on evaluating skin basalioma incidence (Bencko et al., 1999) in a database collected within our population-based study. Beginning in the mid-1970s, the study covered the entire population of the Prievidza district, Central Slovakia, with the primary goal of following up the incidence of all types of malignancies in this area. Our study attempted to obtain a complete detailed register of all the malignant tumors within an administrative unit of a population of about 125,000. This project was feasible due to our previous national health care system, which operated in this country. Each cancer patient or any person suspected of any malignancy was referred to the district oncologist who was responsible for the final diagnosis and therapy of the patient. Originally, our intention was to perform a 10-year study. However, the data collection efforts and the comprehensive nature of the health care system permitted extending this study to 15 years. We are continuing in collaboration with the local public health authorities and Department of Oncology of the Bojnice General Hospital to add one more 5-year period (1992–1996). The fourth 5-year period is not available and, therefore, this paper covers

only the three 5-year intervals. The study was initiated in 1976. The results of the first year were eliminated as the system of data collection and trials of how our questionnaire was constructed and implemented were fine-tuned. The questionnaire covered basics of personal, family, residential and occupational history.

The district was split into two areas marked off by a 7-km circle around the power-plant burning coal with high arsenic content. This circle was established using biological monitoring of human exposure within the particular locality. The exposure rates were established by analysis of hair and urine samples for arsenic content.

To describe the human exposure in environmental settings arsenic determination was carried out on hair, urine, and blood samples taken from groups of 10-year-old boys, each group numbering 20 to 25 individuals, residing in the region polluted by arsenic (Bencko, 1995). The samples were taken from the boys living at various residential places up to approximately 30 km away from the source of emissions. In all the materials examined, elevated concentrations of arsenic were found.

The criterion of higher exposure was arsenic content exceeding, on the average, hair concentrations of 3 μg/g of arsenic. About two-tenths of the district population under study live in a 7-km radius of the exposed region. Values up to 1 μg/g are considered normal (WHO/IPCS, 1981; Jacobson-Kram et al., 1984; ATSDR, 1998; WHO, 2000). For example, the population in Prague showed approximately 0.2 μg/g, which is less than one-tenth of the mean value, which predominated in this previously heavily emission-loaded area near Prievidza.

On the basis of the results obtained, the most advantageous material for estimation of non-occupational exposure and especially to demonstrate environmental pollution seems to be hair, in spite of some problems with the decontamination procedure involved. The results corresponded to the theoretical ideas on spreading of emissions from elevated sources in the open air and tend to establish the applicability of arsenic determination in the hair as suitable means for monitoring contamination of the environment by arsenic. Considerable variability among individual arsenic values in the hair makes group examination a necessity. The same applies to the blood and urine sampling, which is complicated by several technical difficulties concerning sampling and storage of the collected samples. Levels in urine reflect the quantities of arsenic inhaled or ingested after their absorption into the blood, and give a more realistic picture of possible total daily intake during recent days. In the region polluted by emissions arising from arsenic-contaminated coal, elevated values of arsenic were detected in autopsy samples as well (Bencko, 2000).

RESULTS AND DISCUSSION

Preliminary analysis of the database (Tables 1 and 2) collected during 15 years (three 5-year intervals) of 844 lung cancer cases suggests a permanent increase of lung cancer incidence in both the most polluted part of the district and the rest of it. The incidence of lung cancer is markedly influenced by exposure to arsenic in occupational settings as can been seen from Table 3.

Measurements, conducted quite recently (Fabiánová et al., 1994), have confirmed that the significantly increased arsenic concentrations exceeding the established hygienic limit values (MAC) for arsenic in occupational settings occur mainly during boiler-cleaning operations. Considering, however, the relatively long period of latency, so frequently described in arsenic-caused cancers, we may assume that the changed tumor mortality pattern was a result of arsenic exposures during the years characterized by the much less favorable hygienic conditions at the workplaces from the end of the 1950s to the mid-1970s.

As the result of radical reduction of emissions the main interests now are the late effects of the previous occupational and environmental exposure to arsenic in the former

TABLE 1

Lung cancer incidence in population living in the vicinity of the power-plant burning coal of high arsenic content and in the rest of the district (males only)

	1977–1981		1982–1986		1987–1991		1977–1991	
	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)
Absolute number	35	176	41	200	49	257	125	633
Expected number	31.43		33.73		39.67		105.21	
Nonstandardized rate	77.83	65.16	97.66	69.78	132.80	84.89	100.93	73.65
Age standardized rate	73.51	65.83	86.43	71.13	107.47	87.35	89.09	75.07
Person-years	(44969)	(270119)	(41980)	(286619)	(36898)	(302734)	(123847)	(859472)

Statistical parameters (confidence interval $P = 0.1$)

		Min.	Max.		Min.	Max.		Min.	Max.		Min.	Max.
Ratio of standardized rates	1.12	0.83	1.52	1.22	0.92	1.62	1.23	0.95	1.59	1.19	1.01	1.40
Mantel-Haenszel estimate	1.11	0.82	1.51	1.22	0.92	1.61	1.23	0.96	1.60	1.19	1.01	1.40
Chi-square	0.34	NS		1.30	NS		1.84	NS		3.10	S	$P < 0.1$
Standardized morbidity ratio	1.11			1.22			1.24			1.19		

TABLE 2

Lung cancer incidence in population living in the vicinity of the power-plant burning coal of high arsenic content and in the rest of the district (females only)

	1977–1981		1982–1986		1987–1991		1977–1991	
	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)
Absolute number	3	19	6	28	3	27	12	74
Expected number	3.44		4.71		4.11		12.33	
Nonstandardized rate	6.80	7.06	14.45	9.75	8.09	8.83	9.78	8.58
Age standardized rate	6.35	7.16	12.99	9.95	6.36	9.07	8.60	8.77
Person-years	(44111)	(269061)	(41518)	(287148)	(37073)	(305853)	(122702)	(862062)

Statistical parameters (confidence interval $P = 0.1$)

		Min.	Max.		Min.	Max.		Min.	Max.		Min.	Max.
Ratio of standardized rates	0.89	0.32	2.47	1.31	0.62	2.75	0.70	0.26	1.91	0.98	0.59	1.64
Mantel-Haenszel estimate	0.88	0.32	2.43	1.28	0.61	2.68	0.73	0.27	1.98	0.97	0.58	1.63
Chi-square	0.05	NS		0.30	NS		0.28	NS		0.01	NS	
Standardized morbidity ratio	0.87			1.28			0.73			0.97		

TABLE 3

Lung cancer incidence in workers of the power-plant burning coal of high arsenic content (ENO) and in the rest of the district (males only)

	1977–1981		1982–1986		1987–1991		1977–1991	
	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)	Exposed cases (p-years)	Rest of dist. cases (p-years)
Absolute number	3	208	5	236	10	296	18	740
Expected number	4.90		4.73		5.02		14.67	
Nonstandardized rate	44.96	67.44	67.69	73.47	136.97	89.07	84.27	76.93
Age standardized rate	31.76	67.57	159.49	73.26	988.94	88.63	196.86	76.75
Person-years	(6672)	(308416)	(7387)	(321213)	(7301)	(332331)	(21360)	(961960)

Statistical parameters (confidence interval $P = 0.1$)

		Min.	Max.		Min.	Max.		Min.	Max.		Min.	Max.
Ratio of standardized rates	0.47	0.16	1.38	2.18	0.64	7.43	11.16	3.89	32.05	2.56	1.28	5.10
Mantel-Haenszel estimate	0.61	0.23	1.60	1.07	0.51	2.24	2.00	1.18	3.41	1.23	0.83	1.83
Chi-square	0.72	NS		0.02	NS		4.84	S	$P < 0.05$	0.78	NS	
Standardized morbidity ratio	0.61			1.06			1.99			1.23		

heavily polluted region including risk assessment aspects of the situation (Bencko et al., 2000; Fabiánová et al., 2000).

CONCLUSION

Currently, we are performing meta-analysis of the database of the malignant tumors obtained in the population-based epidemiological study within the EXPASCAN, INCO-COPERNICUS project. Future analysis will include all types of malignancies, including kidneys. Lung carcinoma has already been associated with inhalation exposure to arsenic (Pershagen et al., 1977; Léonard and Lauwerys, 1980; WHO/IPCS, 1981; IARC Monographs, 1982; Jacobson-Kram et al., 1984; Pershagen, 1985; Ishinishi et al., 1986; ATSDR, 1998). In spite of known problems with exposure assessment due to cigarette smoking we carefully registered smoking habit in all cases included in our present study. The main objective of our present activity is to assess the exposure as precisely as possible in lung cancer cases, especially taking into account exposure to arsenic and smoking habit of patients. The smoking was carefully registered in all our cancer patients which gives us a chance to study potential synergism between arsenic exposure and smoking in the induction of lung cancer (Welch et al., 1982; Jarup and Pershagen, 1991; Hertz-Picciotto et al., 1992) and other cancer sites. General data about cigarette smoking of the population were collected within the CINDY project during the nineties. The available data are analyzed in collaboration with the district and regional Institutes of Public Health in Prievidza and Banská Bystrica, respectively.

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Arsenic Exposure and Health Effects IV
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Arsenic Ingestion and Health Effects in Bangladesh: Epidemiological Observations

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ABSTRACT

This paper discusses exposure to arsenic in drinking water and its relation to some novel chronic health effects, namely diabetes mellitus and hypertension, as well as to the well-known skin manifestations. Four villages in Bangladesh were identified as mainly dependent on wells potentially contaminated with arsenic. A total of 1481 subjects in these villages, ≥30 years of age and of both genders, were identified as exposed to arsenic. All these individuals were interviewed and examined for blood pressure, skin lesions, and glucosuria. Arsenic levels in drinking water ranged from non-detectable to 2040 μg/l and exposure was expressed as time-weighted concentrations. Urine samples from the study subjects were tested by glucometric strip and positive tests were considered to be cases of glucosuria. Hypertension was defined as a systolic blood pressure ≥140 mm Hg in combination with a diastolic blood pressure of ≥90 mm Hg. Out of the 1481 exposed individuals, 430 exhibited keratosis, i.e., the overall prevalence of skin lesions was 29% in the studied villages. After adjustment for age, sex, and BMI, the prevalence ratio (or risk) of 1.6 was obtained for hypertension in exposed subjects with skin lesions, as compared to those lacking lesions. Likewise the prevalence ratio of 1.8 was found for glucosuria in exposed subjects with skin lesions after adjustment by age, sex, and BMI. Significant trends in risk ($P < 0.001$) were observed between exposure to arsenic and glucosuria and hypertension, respectively, which strengthens the likelihood of a causal association of these conditions with arsenic exposure.

Keywords: arsenic; diabetes mellitus; drinking water; exposure; hypertension; skin lesion

INTRODUCTION

Arsenic is a naturally occurring element in the environment, both in organic and inorganic forms. The use of drinking water with elevated arsenic concentrations, primarily from natural contamination, has been the main source of environmental exposure, but there is also industrial use of arsenic causing exposure. The heaviest occupational exposure to arsenic has traditionally been in copper smelters, but arsenic is also used in the microelectronics industry, in glass production, in pesticides, for wood impregnation and in tanning.

Because arsenic is a recognised carcinogen, the scientific and regulatory focus has been on cancer risks. Studies have also examined effects on atherosclerotic diseases and peripheral vascular diseases in different geographical areas and under varying exposure conditions. In this paper, we reconsider data from an epidemiological study that was conducted in Bangladesh (Rahman et al., 1999a,b; Tondel et al., 1999) and present some additional evaluations regarding exposure to arsenic through drinking water, skin lesions and the prevalence of hypertension and glucosuria.

A major proportion of the tubewells in Bangladesh have been contaminated with arsenic above the permissible limit of the World Health Organisation (Ahmad et al., 1997, 1999; Dhar et al., 1997; Biswas et al., 1998; Rahman et al., 1998, 1999a,b; Milton and Rahman, 1999; Tondel et al., 1999; Chowdhury et al., 2000). Similar contamination, although on different levels, has been reported from other parts of the world, e.g., Argentina, Chile, India, Mexico, Taiwan and the United States (IARC, 1980, 1987; WHO, 1981).

Chronic ingestion of inorganic arsenic causes characteristic skin lesions, such as keratosis, hyperpigmentation and hypopigmentation, as observed in populations in Bangladesh (Milton and Rahman, 1999; Tondel et al., 1999) and elsewhere (Guha Mazumder et al., 1998). These skin lesions have also been considered to be an indicator of high exposure, and to constitute a pre-cancerous condition. Advanced keratosis is painful and results in disfigurement that can lead to social isolation. The high exposure to arsenic poses a public health problem in Bangladesh that has only recently attracted interest from the world. This situation seems to be one of the largest arsenic calamities of the world, with 30–70 million people in Bangladesh currently consuming arsenic-contaminated water (Chowdhury et al., 2000; Smith et al., 2000).

METHODOLOGY

Study Area and Subjects

Based on existing surveys of arsenic in drinking water, the populations in four villages were selected for study. The number of wells of these specific villages was not known, but from the available arsenic measurements, more than 50% of the wells were known to have concentrations of more than 50 μg/l.

All persons who had lived in the study areas throughout their lives were considered eligible for the study. A total of about 4000 individuals were initially identified for this study had lived in the study area their entire lives, but many of these individuals were younger than 30 years and therefore of less primary interest to study as unlikely to have developed the disorders at issue here. The remaining part of the population that was eligible for an interview and examination encompassed 1794 persons, all of whom had used the same well since it was drilled. Out of this eligible population, a total of 1595 individuals agreed to participate. Interviews and medical examinations were performed in the participants' home. Blood pressure was measured in accordance with the WHO protocol (Rose et al., 1982), and urine samples were collected for examining glucosuria. The information obtained indicated that 114 of the 1595 participants were unexposed, that is, they used water from wells with undetectable arsenic levels. The remaining 1481 individuals above the age of 30 years had all used arsenic containing water.

Case Definition (Hypertension and Glucosuria)

Hypertension was defined as a systolic blood pressure ≥140 mm Hg in combination with a diastolic blood pressure of ≥90 mm Hg (JNCV, 1993). Blood pressure was taken after rest and relaxation for at least 15 min in sitting position according to the protocol recommended by the World Health Organisation (Rose et al., 1982).

Urine samples were obtained from all exposed subjects at the time of the health examination. The samples were analyzed by means of a glucometric strip (BM-Test Glucose, Boehringer Mannheim GmbH, Mannheim, Germany). People with positive tests were classified as glucosuric case.

Exposure Assessment

In this supplementary analysis of our data considering the occurrence of hypertension and glucosuria, the exposure is simply taken as the presence of skin lesions. In our previous studies (Rahman et al., 1998, 1999a,b), time-weighted mean lifetime arsenic exposure levels were calculated for each subject, however, and the resulting estimates were then divided into three categories, corresponding to time-weighted arsenic concentrations of <500, 500–1000 and >1000 μg/l. Here we also include some results stemming from the previous studies of this same population.

Statistical Analysis

Subjects were stratified by age (30–39, 40–49, 50–59, and >60 years) and sex; data were also available permitting an evaluation of the influence from body mass index (BMI; using the categories <19, 19–22, and >22). Prevalence ratios, either crude or Mantel–Haenszel weighted (MH-PR), with 95% confidence intervals (CI) and a test for the trend were calculated (EPI 6, Dean et al., 1988).

RESULTS

Table 1 shows some background variables in for those with and without skin lesions. Out of the 1481 exposed subjects who participated in this study, 29% showed at least one sign of skin lesion with a higher prevalence for men than women, respectively ($P < 0.05$). In a previous analysis of this study, material by loglinear regression a significant trend in arsenic levels was obtained both for males and females with a relative risk of 1.6 per 1000 μg/l (CI 1.2–1.9) and 1.4 per 1000 μg/l (CI, 1.0–1.9), respectively (Tondel et al., 1999).

The crude prevalence ratio (or risk) was 1.6 for hypertension in exposed subjects with skin lesions, as compared to those lacking lesions; that estimate remained the same after adjustment for age, and sex (Table 2). The risk was also essentially unchanged after adjustment for age and sex only, indicating that BMI had no effect. With the estimates applied, women had a slightly higher risk of developing hypertension, compared to men (MH-PR for men 1.5 versus 1.8 for women).

TABLE 1

Distribution of some characteristics of all exposed individuals

Variables	Skin lesion		No Skin lesion		*P*
	Mean	SD	Mean	SD	
Age (years)	43.23	10.8	43.1	10.6	0.805
Weight (kg)	50.8	7.7	49.7	7.2	0.500
Height (cm)	157	6.3	157	6.0	0.254
Years of exposure	7.44	5.4	8.7	4.9	0.009

TABLE 2

Distribution of cases of hypertension and non-case subjects along with the crude prevalence ratio and Mantel–Haenszel prevalence ratio (MH-PR), adjusted for age, sex, and considering the presence of skin lesions as an indicator of arsenic exposure

Age (years)	Skin lesions (Yes/No)	Hypertension			
		Men		Women	
		Non-cases	Cases	Non-cases	Cases
30–39	Yes	106	17	52	12
	No	248	26	171	22
40–49	Yes	78	9	42	8
	No	165	18	108	15
50–59	Yes	32	15	19	8
	No	86	18	75	10
>60	Yes	18	4	6	4
	No	55	8	22	4
Total	Yes	234	45	119	32
	No	554	70	376	51
Crude prevalence ratio			1.4		1.8
95% CI			1.0–2.0		1.1–2.6
Crude prevalence ratio (total)				1.6	
95%CI				1.2–2.0	
MH-PR				1.6	
95% CI				1.2–2.0	

The crude prevalence ratio was 1.8 for glucosuria in exposed subjects with skin lesions, as compared to those lacking lesions, and also that estimate remained the same after adjustment for age, sex and BMI. The risk was also essentially unchanged after adjustment for age and sex only, again indicating that BMI had no effect (Table 3). Also in this respect women had a slightly higher risk of developing glucosuria, compared to men (MH-PR for men 1.7 versus 1.9 for women).

DISCUSSION

The findings presented here provide evidence that ingestion of inorganic arsenic in drinking water may results in increases risk of hypertension as well as glucosuria (Table 4). There seemed to be a somewhat higher risk for women to develop hypertension and glucosuria than for men, but the difference was small and whether this represents a true biological effect or a chance phenomenon remains unclear. Although a relationship between ingested arsenic and diabetes mellitus and hypertension has so far only been reported in a limited number of studies from Taiwan, Sweden and now in Bangladesh (Lai et al., 1994; Chen et al., 1995; Rahman and Axelson, 1995; Rahman et al., 1996, 1999a,b), it appears likely that there is a causal relationship.

The main weakness of the current investigation is a cross-sectional design, limited time and small resources in general were available for interviewing and carefully examining each subject included in the study. Some observation bias might likely have occurred during measuring blood pressure, or in the interviews and/or clinical examinations of patients with skin lesions, in so far as the observer could have some idea of the exposure status of the subjects, but not their levels. An observation bias of this kind could likely have created the observed dose–effects relationship, but a fully blind situation is desirable,

TABLE 3

Distribution of cases of glucosuria and non-case subjects along with the crude prevalence ratio and Mantel–Haenszel prevalence ratio (MH-PR), adjusted for age, sex, and considering the presence of skin lesions as an indicator of arsenic exposure

Age (years)	Skin lesions (Yes/No)	Glycosuria			
		Men		Women	
		Non-cases	Cases	Non-cases	Cases
30–39	Yes	100	23	45	19
	No	243	31	164	29
40–49	Yes	71	16	39	11
	No	163	20	103	20
50–59	Yes	31	16	18	9
	No	86	18	73	12
>60	Yes	17	5	4	6
	No	54	9	21	5
Total	Yes	219	60	106	45
	No	546	78	361	66
Crude prevalence ratio			1.7		1.9
95% CI			1.2–2.4		1.3–2.7
Crude prevalence ratio (total)				1.8	
95% CI				1.4–2.2	
MH-PR				1.8	
95% CI				1.4–2.5	

TABLE 4

Prevalence ratios (and 95% confidence intervals) obtained in three studies of diabetes mellitus and/or glucosuria and hypertension in relation to arsenic concentrations in drinking water in Bangladesh

Study and disease	Time weighted arsenic concentrations in μg/l		
	<500	500–1000	>1000
Rahman et al., 1998			
Diabetes mellitus among keratotic subjects	2.6 (1.2–5.7)	3.9 (1.8–8.2)	8.8 (2.7–28.4)
Rahman et al., 1999a *			
Glucosuria among subjects without skin lesions	0.8 (0.4–1.3)	1.4 (0.8–2.3)	1.4 (0.7–2.4)
Glucosuria among subjects with skin lesions	1.1 (0.5–2.0)	2.2 (1.3–3.8)	2.6 (1.4–4.6)
Rahman et al., 1999b *			
Hypertension among subjects with skin lesion	1.2 (0.6–2.3)	2.2 (1.1–4.3)	2.5 (1.2–4.9)

The reference populations are unexposed to arsenic.
* The same population as further considered in this paper.

or nonetheless, any such bias would hardly explain the findings, as any misclassification of the skin lesions would rather have led to a lower magnitude of the prevalence ratios.

A strength of this study is the clear a priori hypotheses that arsenic exposure may lead to an increased risk of both of the conditions at issue, i.e., in view of the earlier findings

in Taiwan (Lai et al., 1994; Chen et al., 1995). Study subjects who participated in the health examination in Bangladesh comprised a higher proportion of men with a slightly higher mean age than for the women (43.2 and 43.1 years, respectively). Men were found to have arsenic-related skin lesions slightly more often than women under seemingly equal exposure to arsenic in drinking water, both sexes sharing the same environment. The significant male-to-female ratio of 1.2 : 1 for skin lesions in our data can perhaps be explained by the assumption that the men had more extensive exposure to arsenic, because, compared to the women, they performed heavier outdoor work and therefore perspired more and drank more water. Alternatively, some undetected selection phenomenon may be responsible for this male-to-female ratio. The latency for arsenic-caused skin lesions, in particular for keratoses, is typically in order of 10 years but appears to be dose dependent (Guha Mazumder et al., 1998). These aspects need further exploration, however.

A central but problematic issue in epidemiological studies concerns the assessment of risk in relation to exposure. A WHO task group studied skin cancer by applying a linear threshold model and estimated that lifetime exposure to 200 μg/l arsenic in drinking water would result in a 5% cumulative incidence. It has also been suggested that several years of ingestion of about 1000 μg of arsenic per day may cause skin effects, and other estimations have indicated that chronic intake of about 10 μg/kg/day or more may result in dermatological conditions and other signs of arsenic toxicity. Our data seem to be in reasonable agreement with this latter estimate when considering the weight of the subjects in the study population and their likely intake of water.

CONCLUSIONS

The health effects that result from the ingestion of arsenic-contaminated drinking water manifest slowly. The future burden of arsenic-caused skin cancer in Bangladesh is uncertain, but large numbers of skin cancers have been reported in Taiwan, and elsewhere (IARC, 1980, 1987; WHO, 1981). In other countries studied, the main causes of death due to chronic ingestion of arsenic in drinking water are internal cancers; skin cancers are not usually fatal with appropriate treatment. Dramatic increases in mortality rates from internal cancers have been reported in Taiwan (Chen et al., 1985, 1988, 1992), and Chile (Smith et al., 1998). Long-term drinking of water containing 500 μg/l of arsenic may ultimately result in 1 in 10 persons ultimately dying from arsenic-caused cancers including lung, bladder and skin cancer (NRC, 1999).

Nonetheless, the strength of the current findings in terms of the high prevalence ratios, along with the plausibility of finding corresponding from Taiwan, that diabetes mellitus and hypertension are effects that may indeed result from ingestion of inorganic arsenic. However, the mechanism underlying the ability of inorganic arsenic to induce these disorders is still unclear. Various sources of exposure should be taken into consideration in further investigations of the indicated effects of arsenic.

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Arsenic Exposure and Health Effects IV
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Occurrence of Cancer in Arsenic Contaminated Area, Ronpibool District, Nakorn Srithmmarat Province, Thailand

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ABSTRACT

Arsenic contamination in Ronpibool district, Nakorn Srithammarat, the southern province of Thailand, has been discovered more than 10 years ago. However, the evidence of human cancer in this population has not been thoroughly studied. This study aimed to assess the prevalence of cancer from two main methods: (a) analysis of secondary data from 5-years (1994–1998) cancer registry of the Prince of Songkhla University, (b) investigation of causes of death using verbal autopsy and hospital records in 1998. When comparing 5-year prevalence of cancer from the cancer registry, Ronpibool district (2.2 per 1000 population) ranked fifth among the 23 districts. When comparing cancer sites among the first 5 high-prevalence districts, Hausai (3.19/1000), Lansaka (2.29/1000), Phomkiri (2.23/1000), Kanom (2.23/1000) and Ronpibool (2.20/1000), lung cancer was the highest in Hausai, Lansaka and Phomkiri, while cancer of the oral cavity (includes cancer of tongue, palate, mouth, saliva gland, parotid gland and tonsil) was found the highest in Kanom. For Ronpibool district lymphoma (includes Hodgkin's, follicular node, diffuse non-Hodgkin's, T-cell and other lymphoma) and breast cancer had the highest proportion (13.5followed by lung cancer (10.2districts, however among the top five districts, Ronpibool had the highest proportion of skin cancer (3.4in females in four of these five Districts.

The study to verify causes of death in Nakorn Srithammarat province in 1998 found a different pattern. The highest death rate from cancer was in Kanom District (120.5 per 100,000), followed by Chalermprakiet (69.7), Hausai (56.7) and Lansaka (57.4). Ronpibool district ranked 12 with a cancer death rate of 31.1 per 100,000. In every District, most cancer patients died at the age of 45 and over, and males had a higher number of deaths than females in these age groups. Details of the evidences will be discussed.

Keywords: Ronpibool District; Thailand; cancer prevalence; causes of death from cancers; cancer registry; verbal autopsy

BACKGROUND

Ronpibool district is situated approximately 70 km south of Nakorn Sri Thammarat Province, in the southern part of Thailand. Total population in this District is around 23,000 in 1998. The geology survey in Ronpibool District has found that potential sources of arsenic contamination in the mining areas were from: high-grade arsenopyrite waste piles in bedrock mining localities, sub-ore grade waste-rock piles, sulfide-rich wastes from ore-dressing plants, disseminated sulfide waste from small-scale prospecting and flotation activities, and alluvial tin workings. Total arsenic concentrations in surface waters were found in the range of 4.8–583 μm/l. The highest arsenic concentrations recorded in surface waters occur in the mid-reaches of the main river (555 μg/l) extending downstream of Ronpibool town (583 μg/l) for a distance of at least 4 km (541 μg/l). Analytical arsenic speciation data were collected for all surface waters, samples holding more than 100 μm/l total arsenic. Significant concentrations of arsenate (As(III)) were detected in several of the most arsenic-rich samples (28.4, 25.6 and 24.9 μg/l), although arsenite (As(V)) remained the dominant species, with more than 92% of total As. It was found that health was affected through extensive arsenic contamination in soil and natural water resources in Ronpibool district. Even though arsenic contamination in this area was discovered for more than 10 years, the evidence of human cancer in this population has not been clearly studied.

The Prince of Songkhla University is the cancer registry center in the southern part of Thailand. Most cancer cases in the south seek treatment and are recorded in the cancer registry. This study aimed to assess the prevalence of cancer from two main sources:

TABLE 1

Rates of major cancers in Nakorn Srithammarat Province by district, cancer registry 1994–1998

Rank	District	Prevalence of cancer		Total population
		number	rate/1000	
1	Hausai	118	3.19	37,004
2	Lansaka	92	2.29	40,069
3	Phomkiri	77	2.23	34,556
4	Kanom	61	2.23	27,375
5	Ronpibool	149	2.20	67,613
6	Tampannara	35	2.10	16,656
7	Pakpanang	236	1.98	119,383
8	Muang	514	1.92	267,526
9	Tungsong	261	1.78	146,410
10	Chienyai	85	1.78	47,845
11	Bangkhun	34	1.69	20,037
12	Tasala	172	1.63	105,662
13	Naborn	45	1.62	27,749
14	Srichon	122	1.52	80,417
15	Cha-uad	121	1.48	81,672
16	Chalermprakiet	52	1.45	35,837
17	Phipoon	40	1.41	28,255
18	Praprom	57	1.41	40,367
19	Changklang	44	1.34	32,887
20	Chawang	40	1.32	69,698
21	Juraporn	41	1.27	32,264
22	Tungyai	70	1.10	63,715
23	Noppitum	30	1.09	27,463
24	Provinces	2,548	1.75	1,450,460

(a) data obtained from 5 years (1994–1998) cancer registry by the Prince of Songkhla University; (b) causes of death data using verbal autopsy and hospital records in order to verify the leading causes of death in 1998.

MATERIAL AND METHODS

The first data set was drawn from the study 'Verifying causes of deaths in 5 provinces and 4 areas in Bangkok of Thailand, 1997–1998', conducted by Choprapawon et al. (2000). Close relatives of all deaths in these selected provinces and areas were interviewed by health personal using 'verbal autopsy' and then three medical doctors gave causes of deaths from the interviewed data and medical records. The second data set was derived from the cancer registry of the Songkhla Nagarindth hospital, Prince of Songkhla University. Prevalences of cancer from both data sets were compared in order to estimate the occurrence of cancer in populations with arsenic exposure.

RESULTS

When comparing 5-year (1994–1998) prevalence rates of cancer from the cancer registry, Ronpibool District (2.2 per 1000 people) ranked fifth among 23 districts, the first 5 high-prevalence districts being Hausai (3.19/1000), Lansaka (2.29/1000), Phomkiri (2.23/1000), Kanom (2.23/1000) and Ronpibool (2.20/1000) as shown in Table 1. The rate of lung cancer

TABLE 2

Number and percent of cancer by organ in the 5 highest ranking districts, Nakorn Srithammarat province, cancer registry 1994–1998

Site of cancer	Hausai		Lansaka		Phomkiri		Kanom		Ronpibool	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Lung	15	12.7	12	13.0	16	20.8	8	13.1	6	10.2
Cervix	14	11.8	12	13.0	7	9.1	8	13.1	4	6.8
Breast	10	8.5	5	5.4	7	9.1	3	4.9	8	13.5
Colon–rectum	9	7.6	5	5.4	1	1.3	2	3.3	0	0
Lymphoma [a]	9	7.6	3	3.3	2	2.6	2	3.3	8	13.5
Bladder–kidney	8	6.8	1	1.1	4	5.2	3	4.9	2	3.4
Leukemia [b]	6	5.1	6	6.5	4	5.2	1	1.6	4	6.8
Oral cavity [c]	5	4.2	9	9.8	8	10.4	13	21.3	5	8.5
Larynx	5	4.2	2	2.2	0	0	3	4.9	3	5.1
Liver	5	4.2	5	5.4	3	3.9	2	3.3	1	1.7
Ovary–corpus	4	3.3	4	4.3	2	2.6	1	1.6	2	3.4
Nasopharyn [d]	4	3.3	9	9.8	8	10.4	6	9.8	3	5.1
Prostate–penis	4	3.3	4	4.3	2	2.6	2	3.3	3	5.1
Thyroid	4	3.3	2	2.2	2	2.6	0	0	1	1.7
Connective	3	2.5	0	0	1	1.3	2	3.3	1	1.7
Multiple myeloma	2	1.7	0	0	0	0	0	0	0	0
Skin	1	0.8	2	2.2	2	2.6	1	1.6	2	3.4
Unspecified	3	2.5	5	5.4	4	5.2	2	3.3	4	6.8
Others	7	5.9	6	6.5	4	5.2	2	3.3	2	3.4
Total	118	100	92	100	77	100	61	100	59	100

[a] Lymphoma including Hodgkin's, follicular node, diffuse non-Hodgkin's, T-cell, other.
[b] Leukemia including lymphoid, myeloid, other.
[c] Oral cavity including tongue, palate, mouth, saliva gland, parotid gland, tonsil.
[d] Nasopharyn including nasopharynx, pharynx, esophagus.

TABLE 3

Number of cancer types by sex, top 5 districts, cancer registry 1994–1998

Site of cancer	Hausai		Lansaka		Phomkiri		Kanom		Ronpibool	
	M	F	M	F	M	F	M	F	M	F
Lung	8	7	11	1	14	2	4	4	10	3
Cervix	0	14	0	12	0	6	0	8	0	15
Breast	0	10	0	5	0	7	0	3	0	10
Colon–rectum	5	4	3	1	1	0	2	0	6	3
Lymphoma [a]	3	6	2	1	1	1	0	2	11	5
Bladder–kidney	6	2	0	1	3	0	3	0	4	0
Leukemia [b]	3	3	5	2	4	1	1	0	6	4
Oral cavity [c]	5	0	3	6	5	3	10	3	8	4
Larynx	5	0	2	0	0	0	2	1	5	0
Liver	4	1	4	1	3	0	2	0	2	1
Ovary–corpus	0	5	0	4	0	2	0	1	0	9
Upper GI [d]	2	2	7	2	8	0	6	0	10	6
Prostate–penis	4	0	4	0	2	0	2	0	4	0
Thyroid	1	3	0	2	0	2	0	0	1	1
Connective	1	2	0	0	1	0	0	2	2	1
Multiple myeloma	1	1	0	0	0	0	0	0	0	0
Skin	1	0	2	0	1	1	0	1	5	0
Unspecific	1	2	4	1	3	1	1	1	2	2
Others	1	5	1	0	1	1	1	1	7	2
Total	51	67	48	39	47	27	34	27	83	66
M : F	0.76 : 1		1.23 : 1		1.74 : 1		1.25 : 1		1.25 : 1	

[a] Lymphoma including Hodgkin's, follicular node, diffuse non-Hodgkin's, T-cell, other.
[c] Leukemia including lymphoid, myeloid, other.
[c] Oral cavity including tongue, palate, mouth, saliva gland, parotid gland, tonsil.
[d] Upper GI including nasopharynx, pharynx, esophagus, stomach, duodenum.

was highest in Phomkiri District (20.8% of all types), followed by Kanom, Lansaka and Hausai, while cancer of the oral cavity (including cancer of tongue, palate, mouth, saliva gland, parotid gland and tonsil) was highest in Kanom (Table 2). In Ronpibool District, lymphoma (including Hodgkin's, follicular node, diffuse non-Hodgkin's, T-cell and other lymphoma) and breast cancer were highest (13.5% of all sites), followed by lung cancer (10.2%). Few cases of skin cancer were reported in most districts; however, among the top five districts, Ronpibool had highest proportion of skin cancer (3.4%). The rate of cancer in males was higher than in females in four of these five districts. In Ronpibool, all skin cancer patients were males (Table 3).

Results from the study to verify causes of death in Nakorn Srithammarat Province in 1997–1998 showed a different pattern. The highest death rate from cancer was in Kanom District (120.5 per 100,000), followed by Chalermprakiet (69.7), Hausai (56.7) and Lansaka (57.4). Ronpibool District ranked 12 with a cancer death rate of 31.1 per 100,000. In every district, most cancer victims died at the age of 45 and older and for men the number of deaths in this age group was higher (Table 4).

DISCUSSION AND RECOMMENDATION

There is some evidence from studies in Taiwan, Japan, England, USA, Argentina and Chile (Smith et al., 1999) that ingestion of arsenic can cause a variety of cancers (bladder, kidney, liver and lung) other than skin cancer. This study aimed to find some evidence

TABLE 4

Number and rate of cancer deaths by age and sex and district, Nakorn Srithammarat province, 1998 (rate per 100,000 people)

District	Both sexes		5–14		15–24		25–44		45–59		60–74		75+	
	number	rate	M	F	M	F	M	F	M	F	M	F	M	F
Kanom	33	120.5	0	0	0	0	3	0	10	4	9	2	4	1
Chalerm	25	69.7	0	0	0	0	0	2	5	3	7	2	3	1
Hausai	21	56.7	0	0	0	1	1	3	3	4	3	2	2	2
Lansaka	23	57.4	0	0	0	0	0	1	5	5	3	0	7	1
Chawang	39	55.9	0	0	1	0	3	8	0	2	8	4	7	6
Tampanra	7	42.0	0	0	0	0	0	0	2	1	2	1	0	1
Tasala	43	40.7	0	0	0	0	3	2	7	4	14	8	3	2
Bangkhun	8	39.9	0	0	0	0	0	1	3	1	1	0	2	1
Phipoon	10	35.4	0	0	0	0	0	1	2	0	2	4	0	1
Chienyai	16	33.4	0	0	0	0	1	0	4	0	5	3	2	1
Muang	86	32.1	0	1	1	0	4	4	14	10	23	14	14	1
Ronpibool	21	31.1	0	0	0	0	1	4	0	1	5	4	4	2
Tungsong	44	30.0	0	1	1	0	2	2	3	5	9	11	7	3
Praprom	12	29.7	0	0	0	0	0	1	4	0	6	0	1	0
Cha-uad	20	24.5	0	0	1	0	2	1	2	3	8	3	2	0
Srichon	18	22.4	0	0	0	0	3	0	0	3	0	4	0	8
Naborn	4	14.4	0	0	0	0	0	0	1	0	0	0	3	0
Pakpanang	5	4.2	0	0	0	0	1	1	0	0	0	0	2	1
Phomkiri	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Changkang	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Juraporn	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tungyai	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Noppitum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	435	28.75	0	2	4	1	24	31	65	46	105	62	63	32

of increasing cancer risk among the residents in Rompibool District, who have ingested drinking waters markedly contaminated by arsenic for more than 70 years (Choprapawon and Rodcline, 1997). However, the level of ingestion was probably low, so the prevalence of cancer among the villagers even in the area of high arsenic contamination is quite the same as for other areas in the same province. Only skin cancer was reported as higher in Ronpibool District. Unlike the study of Tseng et al. (1968), the cancer registry in the university hospital of the south of Thailand is a passive case detection system, so the non-fatal cancer, especially skin cancer, remained undiagnosed during the whole life of the patient. The case rate of skin cancer may be underestimated. The study of causes of death also found no evidence of increasing death rate from cancer. It is recommended that further cohort studies must be carried out in order to show whether arsenic poisoning in this area might be increasing cancer risk among this population. Another interesting question is whether arsenic contamination in this area might have non-carcinogenic effects.

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Arsenic Exposure, Null Genotypes of Glutathione S-Transferase M1, T1 and P1, and Risk of Carotid Atherosclerosis among Residents in the Lanyang Basin of Taiwan

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ABSTRACT

In order to evaluate the synergistic effects of arsenic exposure through drinking water and genetic polymorphisms of GST M1, T1, and P1 on the risk of carotid atherosclerosis, a total of 605 residents who participated physical examination of arsenic-induced health effects held in Lanyang Basin were recruited as study subjects. A standardized personal interview based on a structured questionnaire was carried out to obtain information on duration of well water consumption, sociodemographic characteristics, cigarette smoking, alcohol drinking, as well as personal and family history of hypertension, diabetes and cerebrovascular disease. Well water samples were collected during the home interview. Hydride generator combined with flame atomic absorption spectrometry was used to determine the arsenic concentration in these samples. Whole blood samples were collected and centrifuged to separate buffy coat. DNA was then purified from buffy coat and stored at −20°C until genotyping. PCR was used for genotyping GSTM1 and GSTT1. PCR-RFLP was also carried out to determine the genetic polymorphisms of GST P1. Intimal-medial thickness (IMT) of arterial walls was measured by B-mode real-time ultrasound as an index of atherosclerosis. Plaque score was also used as an index of atherosclerosis. Logistic regression analysis was used to estimate age–sex-adjusted odds ratio and 95% confidence interval. Age was a major risk for the development of carotid atherosclerosis, showing a significant dose–response relationship. Compared with the youngest age group, the significant odds ratios of 5.9 and 2.3 were observed for the oldest and middle age groups, respectively. Significant risk for occurrence of carotid atherosclerosis was also observed among hypertension patients with age–sex-adjusted odds ratio of 2.4. Significant age–sex-adjusted odds ratios of risk of carotid atherosclerosis were observed in exposure groups with arsenic concentration in well water greater than 50 μg/l, showing odds ratio of 2.5. Moreover, study subjects with cumulative arsenic exposure greater than 1.0 mg/l-year also had significant age–sex-adjusted risk of developing carotid atherosclerosis. A significant higher age–sex-adjusted odds ratio of 2.0 for the development of carotid atherosclerosis was observed among study subjects with genotypes of W/M and M/M for GSTP1. A significant dose–response relationship was observed between risk of carotid atherosclerosis and arsenic exposure indices of arsenic concentration in well water and cumulative arsenic exposure among study subjects with null genotype of GSTM1, T1 and with W/M or M/M genotype of GSTP1. In conclusion, a significant effect on the risk of carotid atherosclerosis was observed among study subjects with arsenic exposure and with genotypes of W/M and M/M. In addition, significant synergistic effects on the risk for development of carotid atherosclerosis were also found between arsenic exposure and various genotypes of GST M1.

Keywords: arsenic; glutathione S-transferase; atherosclerosis

INTRODUCTION

Arsenic is widely distributed in nature and mainly transported in the environment by water. Humans are exposed to arsenic through water, air, food and medicine mainly through ingestion and inhalation, and rarely through skin absorption. The main source of arsenic exposure for the general population is through ingestion of water containing a high level of inorganic arsenic and through ingestion of seafood containing high levels of organic arsenic. However, organic arsenic is less toxic than inorganic arsenic (World Health Organization, 1981; U.S. Public Health Service, 1989). The tentative MCL for arsenic in drinking water set by the U.S. Environmental Protection Agency is 0.05 mg/l. In the United States, it has been estimated that about 350,000 people may drink water containing more than this level of arsenic (Smith et al., 1992). Arsenic is involved in the development of several cancers in human without showing any organotropism. Inorganic arsenic has been well documented as a human carcinogen of skin and lung (World Health Organization, 1981; International Agency for Research on Cancer, 1987; U.S. Environmental Protection Agency, 1988; U.S. Public Health Service, 1989). Serious epidemiological studies have shown that long-term exposure to arsenic through drinking contaminated well water would increase the risk for development cancers of the skin, liver, nasal cavity, lung, bladder, kidney and prostate in Taiwan (Chen et al., 1988b, 1992; Chen and Wang, 1990; Chiou et al., 1995, 2001).

Inorganic arsenic has also been well documented as one of the major risk factors for black foot disease (BFD), a unique peripheral vascular disease identified in the endemic area of arseniasis located on the southwest coast of Taiwan where residents had used high-arsenic artesian well water for more than 50 years (Tseng et al., 1968; Chen et al., 1988a). Clinically the disease starts with numbness or coldness, and ends with gangrene and spontaneous amputations of one or more affected extremities (Tseng, 1977; Chen et al., 1988a). The pathological types of BFD include arteriosclerosis obliterans (70%) and thromboangitis obliterans (30%) which developed from severe underlying systemic arteriosclerosis (Tseng et al., 1961; Yeh and How, 1963). BFD patients were found to have a significantly increased prevalence of skin hyperpigmentation and hyperkeratosis (Tseng et al., 1968). They also have an elevated mortality from various internal cancers, ischemic heart disease and cerebral infarction (Chen et al., 1988a; Chiou et al., 1995).

The atherogenic effects of arsenic has been well documented. The occupational exposure to inorganic arsenic through inhalation of polluted air from copper smelting and pesticide manufacturing was found to be associated with a moderate, but not statistically significant, excess mortality from ischemic heart disease and cardiovascular disease (CVD) (Enterline and Marsh, 1982; Lee-Feldstein, 1983; Jarp et al., 1989). Ingested inorganic arsenic through drinking water has been related to the development of peripheral vascular disease in Poland, Chile, Mexico, Argentina, Japan and Xinjiang, China (Borgono et al., 1977; World Health Organization, 1981; Hotta, 1989; Chen and Lin, 1994; Engel et al., 1994; Cebrian et al., 1994; Wang and Huang, 1994) and among Moselle vintners exposed to inorganic arsenic through contaminated wine (Grobe, 1976). Current studies have also reported that long-term exposure to arsenic in drinking water was significantly associated with risk for development of ischemic heart disease, cerebrovascular disease and peripheral vascular disease in Taiwan, showing a dose–response relationship (Wu et al., 1989; Chen and Wang, 1990; Tseng et al., 1996; Chen et al., 1996; Chiou et al., 1997). In addition, the association between ingestion of arsenic through drinking water and hypertension and diabetes mellitus has also been reported in Taiwan and Bangladesh (Lai et al., 1992; Chen et al., 1995; Rahman et al., 1998, 1999).

The main arsenic species in artesian well water is arsenate. A substantial fraction of arsenate, which is readily absorbed from the gastrointestinal tract by human, is reduced in the blood to arsenite. Arsenite is methylated mainly in liver to monomethylarsonic

acid (MMA) and dimethylarsinic acid (DMA) (Tam et al., 1979). The methylation process may be recognized as a detoxification mechanism since the methylated metabolites are, in comparison with inorganic arsenic, less reactive with tissue constituents, less toxic, and more readily excreted in the urine (Vahter and Marafante, 1983; Vahter et al., 1986; Lei and Ma, 1998; Vahter, 2000). Arsenic species concentrations in urine have been considered as good biological markers of exposure and methylation capacity. The exact mechanisms of arsenic metabolism and excretion in humans are not well understood. In general, inorganic arsenic and metabolites in human urine contained 10–30% inorganic arsenic, 10–20% MMA, and 60–80% DMA (Buchet et al., 1981a,b)

Glutathione (GSH) S-transferases (GST) are a large family of phase II detoxification enzymes that catalyze the conjugation of reduced glutathione to a wide spectrum of hydrophobic and electrophilic compounds. They play important roles in protection mechanisms against chemical carcinogenesis. There are four subclasses of GST in mammalian cells, namely alpha, mu, pi and theta (Board et al., 1990). The null genotype of GSTM1 has a decreased detoxification capability. It has been linked with an increased risk of cancers of the lung, bladder, breast, colon, multiple skin, meloma, and oral cavity (Zhong et al., 1991, 1993; Bell et al., 1993; Brockmoller et al., 1993; Heagerty et al., 1994; Hung et al., 1997; Rebbeck, 1997). The null genotype of GSTT1 was reported to be associated with an increased cancer risks of the brain and colorectal (Chenevix-Trench et al., 1995; Hung et al., 1997; Rebbeck, 1997). The GSTPi is the most ubiquitous of the human GST family, being expressed in different tissues. It has been found to express in various malignant cells of humans including lung, breast, and ovarian (Lorna et al., 1997). A recent study also reported that GSTP1 genotype may play a role in risk for oral cancer particularly among lighter smokers (Park et al., 2000). Many studies have suggested that GSH might be involved in the initial reduction of arsenate to arsenite and the subsequent oxidative methylation. GSH is a necessary enzyme for arsenic methylation, perhaps through the formation of arsenite that is the preferred arsenic form for methylation, or through conjugation with arsenic (Buchet and Lauwerys, 1985, 1987; Thompson, 1993). Humans with null genotypes of GSTM1, T1, and P1 have been considered to be at a high risk of cancer due to their GSH deficiency. The specific aim of this study is to evaluate the synergistic effects of arsenic exposure through drinking water and genetic polymorphisms of GST M1, T1, and P1 on the risk of carotid atherosclerosis.

MATERIALS AND METHODS

Study Area and Study Subjects

A total of eighteen villages in four townships in the Lanyang Basin in the northeast area of Taiwan which has been described in a previous study (Chiou et al., 1997) were included in the present study. A total of 8102 residents who had been recruited as study cohort between October 1991 and September 1994 were described previously (Chiou et al., 1997). Among them, a total of 605 study subjects who participated physical examination of arsenic-induced health effects held in the study area were recruited to this study.

Data Collection and Determination of Arsenic Content in Well Water

The standardized personal interview was used based on a structured questionnaire and was carried out by four public health nurses who were well-trained in the interview technique and questionnaire details. Information obtained from the interview included the duration of well water consumption, residential history, sociodemographic characteristics, cigarette smoking, alcohol consumption, physical activities, history of sunlight exposure, as well as personal and family history of hypertension, diabetes, cerebrovascular disease, heart disease, and cancers. Well water samples were collected during the interviews at home, acidified with hydrochloric acid immediately and then stored at −20ºC until sub-

sequent assay. Hydride generator combined with flame atomic absorption spectrometry was used to determine arsenic concentration in these samples (Kuo, 1968). The arsenic level was found to range from undetectable (<0.15 μg/l) to 3.59 mg/l. The various exposure indices of arsenic through drinking well water have been described in a previous study (Chiou et al., 1997).

Biospecimen Collection and Laboratory Analysis

Whole blood samples (10 ml) were collected from study subjects using disposable vacuum syringe and needle, centrifuged to separate buffy coat and plasma, and stored at −70ºC for subsequent assay. DNA was purified from buffy coat by the Viogene Blood and Tissue Genomic DNA Miniprep System (Viogene, USA) resuspended in deionized distilled water, and store at −20ºC until genotyping. The method for genotyping GSTM1 and GSTT1 using PCR has been reported previously (Pemble et al., 1994). The primers of GSTM1 were G5 (5′-GAA CTC CCT GAA AAG CTA AAG C-3′) and G6 (5′-GTT GGG CTC AAA TAT ACG GTG G-3′), and the product of PCR showed a 215 bp band in the non-null genotype. The primers of GSTT1 were T1F (5′-TTC CTT ACT GGT CCT CAC ATC TC-3′) and T1R (5′-TCA CCG GAT CAT GGC CAG CA-3′), and the product of PCR showed a 480 bp band in the non-null genotype. For GSTP1, PCR-RFLP was also carried out with PCR primers P105F (5′-ACC CCA GGG CTC TAT GGG AA-3′) and P105R (5′-TGA GGG CAC AAG AAG CCC CT-3′). The product of PCR showed a 176 bp in length. It was then digested with the Bsm AI restriction enzyme, the product of wild (W)/wild (W) ($Ile_{105}Ile$) genotype showed a 176 bp band, the mutant (M)/mutant (M) ($Val_{105}Val$) genotype showed a 91 bp and 85 bp band, and the W/M ($Ile_{105}Val$) genotype had all three bands. The primers of the β-globin gene were added in PCR as an internal control. All laboratory examinations were carried out by technicians without knowing the disease status of the study subjects.

Diagnosis of Carotid Atherosclerosis

Intimal-medial thickness (IMT) of arterial walls was measured by B-mode real-time ultrasound as an index of atherosclerosis (Howard et al., 1993). The measured area in the carotid is a 20 mm segment proximal to the dilation of the carotid bulb, which will be referred to as the common carotid artery (CCA). The plaque score was also used as an index of atherosclerosis. Plaque scores were counted if any plaques were observed in the areas of external carotid arterial (ECA), internal carotid arterial (ICA), carotid bulb, and CCA. The study subjects were diagnosed as carotid atherosclerosis patients by neurologists based on IMT and the plaque score.

Statistical Analysis

To evaluate associations between risk factors and prevalence of carotid atherosclerosis, we used logistic regression analysis to estimate the age–sex-adjusted odds ratio and 95% confidence interval (Breslow and Day, 1980). The statistical significance of each age–sex-adjusted odds ratio was examined by testing the significance of the regression coefficient.

RESULTS

A total of 605 study subjects (289 men and 316 women) were recruited in this study. The distribution of study subjects was as follows: 18.2% <55 years, 42.6% 55–64.9 years and 39.2% >65 years old. Among them, 37.2% were cigarette smokers and 18.7% had a history of alcohol drinking. In addition, 13.2% and 6.3% of them were affected with hypertension and diabetes mellitus, respectively.

Table 1 shows sociodemographic characteristics, life style, and disease status of carotid atherosclerosis patients and controls. Age was a major risk for the development of ca-

TABLE 1

Sociodemographic characteristics, life style and disease status of carotid atherosclerosis patients and controls

Variable	Case No. (%)	Control No. (%)	OR (95% CI) [a]	OR (95% CI) [b]
Age:				
<55	25 (8.9)	85 (26.2)	1.0	1.0 [c]
55–64.9	105 (37.5)	153 (47.1)	2.3 (1.4–3.9)***	2.2 (1.3–3.6)
65+	150 (53.6)	87 (26.7)	5.9 (3.5–9.8)****	5.3 (3.1–9.0)
Sex:				
Females	127 (45.4)	189 (58.2)	1.0	1.0Ss[d]
Males	153 (54.6)	136 (41.8)	1.7 (1.2–2.3)****	1.4 (1.0–1.9)*
Body mass index (kg/m^2):				
<20	47 (17.4)	51 (16.3)	1.0	1.0
20–25.9	127 (47.0)	134 (42.8)	1.0 (0.6–1.6)	1.3 (0.8–2.2)
26+	96 (35.6)	127 (40.8)	0.8 (0.5–1.3)	1.3 (0.8–2.4)
Cigarette smoking:				
No	157 (56.1)	223 (68.6)	1.0	1.0
Yes	123 (43.9)	102 (31.4)	1.7 (1.2–2.4)***	1.1 (0.6–1.9)
Alcohol drinking:				
No	215 (76.8)	276 (85.2)	1.0	1.0
Yes	65 (23.2)	48 (14.8)	1.7 (1.2–2.6)***	1.3 (0.8–2.1)
Hypertension:				
No	149 (76.4)	243 (87.7)	1.0	1.0
Yes	46 (23.6)	34 (12.3)	2.2 (1.4–3.6)***	2.4 (1.4–4.0)***
Diabetes mellitus:				
No	179 (91.8)	255 (92.1)	1.0	1.0
Yes	16 (8.2)	22 (7.9)	1.0 (0.5–2.0)	1.1 (0.5–2.2)

[a] Crude odds ratio; [b] age–sex-adjusted odds ratio; [c] sex-adjusted odds ratio; [d] age-adjusted odds ratio; * $0.05 < P < 0.1$ ** $0.01 < P < 0.05$ *** $0.001 < P < 0.01$; **** $P < 0.001$.

rotid atherosclerosis, showing a significant dose–response relationship. Compared with the youngest age group, significant odds ratios were observed for the oldest and middle age groups (OR = 5.9 and OR = 2.3, respectively). A significant risk for occurrence of carotid atherosclerosis was also observed among hypertension patients with an age–sex-adjusted odds ratio of 2.4. In addition, men, alcohol drinkers, and patients with diabetes mellitus also had a higher but not significant risk for the development of carotid atherosclerosis.

Age–sex-adjusted odds ratios of carotid atherosclerosis for various arsenic exposure groups in different exposure indices of arsenic through drinking well water are shown in Table 2. Significant age–sex-adjusted odds ratios of risk of carotid atherosclerosis were observed both in exposure groups with arsenic concentrations in well water greater than 100 and in those which ranged from 50.1 to 99.9 μg/l. Moreover, study subjects with cumulative arsenic exposures greater than 1.0 mg/l-year also had significant age–sex-adjusted risk of developing carotid atherosclerosis.

The risks of carotid atherosclerosis for genetic polymorphisms of GSTM1, T1 and P1 are illustrated in Table 3. A significantly higher age–sex-adjusted odds ratio of 2.0 for the development of carotid atherosclerosis was observed among study subjects with genotypes of W/M and M/M for GSTP1. However, study subjects with null genotypes of GSTM1 and T1 did not have a higher risk of carotid atherosclerosis.

The cross-tabulation of various arsenic exposure indices and genotype of GSTM1 is shown in Table 4. A significant dose–response relationship was observed between arsenic

TABLE 2

Age–sex-adjusted odds ratio (OR) and 95% confidence interval (CI) of carotid atherosclerosis by various arsenic exposure indices

Variable	Case No. (%)	Control No. (%)	OR (95% CI) [a]	OR (95% CI) [b]
Arsenic concentration in well water (μg/l):				
≤50	50 (17.9)	115 (35.4)	1.0	1.00
50.1–99.9	92 (32.9)	84 (25.8)	2.5 (1.6–3.9)****	2.13 (1.04–4.32)****
100+	138 (49.2)	126 (38.8)	2.5 (1.02–4.02)****	2.13 (1.04–4.32)****
Cumulative arsenic exposure (mg/l-year):				
<1.0	58 (20.7)	101 (31.1)	1.0	1.0
1.0–6.9	150 (53.6)	156 (48.0)	1.7 (1.1–2.5)***	1.8 (1.2–2.8)***
7.0+	72 (25.7)	68 (20.9)	1.8 (1.2–2.9)***	1.9 (1.1–3.0)***
Duration of drinking well water (years):				
<30	52 (18.6)	57 (71.03)	1.0	1.0
30–49	127 (45.4)	167 (47.1)	0.8 (0.5–1.3)	0.9 (0.5–1.4)
50+	101 (36.0)	101 (33.1)	1.1 (0.7–1.7)	0.8 (0.5–1.4)

[a] Crude odds ratio; [b] age–sex-adjusted odds ratio; * $0.05 < P < 0.1$; ** $0.01 < P < 0.05$*** $0.001 < P < 0.01$; **** $P < 0.001$.

TABLE 3

Age–sex-adjusted odds ratio (OR) and 95% confidence interval (CI) of carotid atherosclerosis by genetic polymorphisms of GSTM1, T1 and P1

Variable	Case No. (%)	Control No. (%)	OR (95% CI) [a]	OR (95% CI) [b]
GSTM1				
Non-null	136 (48.7)	139 (42.8)	1.0	1.0
Null	143 (51.3)	186 (57.2)	0.8 (0.6–1.1)	0.9 (0.5–1.0)
GSTT1				
Non-null	134 (48.0)	133 (40.9)	1.0	1.0
Null	145 (52.0)	192 (59.1)	0.8 (0.5–1.0) *	0.7 (0.5–1.0) *
GSTP1				
W/W	178 (63.8)	248 (76.4)	1.0	1.0
W/M	95 (34.1)	71 (21.8)	1.8 (1.3–2.6) ***	2.0 (1.4–3.0) ****
M/M	6 (2.1)	6 (1.8)		

[a] Crude odds ratio; [b] age–sex-adjusted odds ratio; * $0.05 < P < 0.1$; ** $0.01 < P < 0.05$; *** $0.001 < P < 0.01$; **** $P < 0.001$.

concentration in well water and risk of carotid atherosclerosis among study subjects with null genotypes of GSTM1. The age–sex-adjusted odds ratios of carotid atherosclerosis were 2.7 and 3.3 for arsenic concentrations in well water of 50.1–99.9 and >100 μg/l, respectively. Among them, significant risks of carotid atherosclerosis were also found for cumulative arsenic exposures of 1.0–6.9 and >7.0 mg/l-year, showing odds ratios of 2.6 and 3.6, respectively. Though a significant dose–response relationship was not found between arsenic concentration in well water and risk of carotid atherosclerosis for the study subjects with non-null genotypes of GSTM1, significant odds ratios of carotid atherosclerosis were observed among various arsenic concentrations in well water.

The association between genotype of GSTT1 and risk of developing carotid atherosclerosis is shown in Table 5. Significant odds ratios of carotid atherosclerosis were 5.3

TABLE 4

Age–sex-adjusted odds ratio (OR) and 95% confidence interval (CI) of carotid atherosclerosis by genetic polymorphisms of GSTM1, and various arsenic exposure indices

Variable	GSTM1					
	Null			Non-null		
	Case	Control	OR [a] (95% CI)	Case	Control	OR [a] (95% CI)
Arsenic concentration in well water (μg/l):						
≤50	25	55	1.0	24	60	1.0
50.1–99.9	43	39	2.7 (1.4–5.3) ***	49	45	3.0 (1.5–5.9) **
100+	69	45	3.3 (1.8–6.2) ****	70	81	2.3(1.3–4.2) **
Cumulative arsenic exposure (mg/l-year):						
<1.0	23	48	1.0	34	54	1.0
1.0–6.9	76	70	2.6 (1.4–4.8) ***	74	85	1.4(0.8–2.5)
7.0+	37	21	3.6 (1.7–7.6)	35	47	1.2(0.7–2.4)
Duration of drinking well water (year):						
<30	25	16	1.0	27	41	1.0
30–49	61	72	0.5 (0.2–1.0) *	66	94	1.2(0.6–2.2)
50+	50	51	0.4 (0.2–0.9) *	50	51	1.2(0.6–2.4)

[a] Age–sex-adjusted odds ratio; * $0.05 < P < 0.1$; ** $0.01 < P < 0.05$; *** $0.001 < P < 0.01$; **** $P < 0.001$.

TABLE 5

Age–sex-adjusted odds ratio (OR) and 95% confidence interval (CI) of carotid atherosclerosis by genetic polymorphisms of GSTT1, and various arsenic exposure indices

Variable	GSTT1					
	Null			Non-null		
	Case	Control	OR [a] (95% CI)	Case	Control	OR [a] (95%CI)
Arsenic concentration in well water (μg/l):						
≤50	24	57	1.0	25	58	1.0
50.1–99.9	50	32	5.3 (2.6–10.8) ****	42	52	1.8 (0.9–3.4) *
100+	60	44	3.6 (1.9–7.0) ****	78	82	2.2 (1.2–4.0) ***
Cumulative arsenic exposure (mg/l-year):						
<1.0	29	48	1.0	28	54	1.0
1.0–6.9	71	63	2.2 (1.2–4.1) **	79	92	1.6 (0.9–2.8)
7.0+	34	22	2.5 (1.2–5.2) **	38	46	1.7 (0.9–3.3)
Duration of drinking well water (year):						
<30	23	29	1.0	29	28	1.0
30–49	69	65	1.5 (0.7–2.9)	58	101	0.5(0.3–1.0)
50+	42	39	1.1 (0.5–2.4)	58	63	0.6(0.3–1.2)

[a] Age–sex-adjusted odds ratio; * $0.05 < P < 0.1$; ** $0.01 < P < 0.05$; *** $0.001 < P < 0.01$; **** $P < 0.001$.

and 3.6 for arsenic concentrations in well water of 50.1–99.9 and >100 μg/l, respectively, among study subjects with null genotypes of GSTT1. Among them, significant risks of carotid atherosclerosis were also found for cumulative arsenic exposures of 1.0–6.9 and ≥7.0 mg/l-year, showing odds ratios of 2.2 and 2.5, respectively. In addition, non-significantly higher risks of carotid atherosclerosis were found among higher arsenic exposure group and groups with non-null genotypes of GSTT1.

TABLE 6

Age–sex-adjusted odds ratio (OR) and 95% confidence interval (CI) of carotid atherosclerosis by genetic polymorphisms of GSTP1, and various arsenic exposure indices

Variable	GSTP1					
	W/W			W/M or M/M		
	Case	Control	OR[a] (95% CI)	Case	Control	OR[a] (95% CI)
Arsenic concentration in well water (μg/l):						
≤50	29	85	1.0	20	31	1.0
50.1–99.9	60	66	2.7 (1.5–4.9) ****	32	18	4.1 (1.–10.0) ***
100+	89	97	2.7 (1.6–4.5) ****	49	29	3.2 (1.4–7.1) ***
Cumulative arsenic exposure (mg/l-year):						
<1.0	39	78	1.0	18	24	1.0
1.0–6.9	93	120	1.6 (1.0–2.6) *	57	36	2.4 (1.1–5.4) **
7.0+	46	50	1.7 (1.0–3.1) *	26	18	2.1 (0.8–5.1)
Duration of drinking well water (year):						
<30	33	43	1.0	19	14	1.0
30–49	84	126	0.9 (0.5–1.5)	43	41	0.8 (0.4–2.0)
50+	61	79	0.7 (0.4–2.6)	39	23	1.0 (0.4–2.6)

[a] Age–sex-adjusted odds ratio; * $0.05 < P < 0.1$; ** $0.01 < P < 0.05$; *** $0.001 < P < 0.01$; **** $P < 0.001$.
W/W = Wild/Wild; W/M = Wild/Mutant; M/M = Mutant/Mutant.

Table 6 illustrates the association between risk of carotid atherosclerosis and genotype of GSTP1. Study subjects who drank well water containing arsenic levels greater than 50 μg/l and with W/M or M/M genotype of GSTP1 had 2.7 higher risk of developing carotid atherosclerosis compared with those who drank well water containing arsenic concentration equal to and less than 50 μg/l as referent group. Significant age-adjusted odds ratios of 4.1 and 3.2 for the development of carotid atherosclerosis were also found for arsenic concentrations in well water of 50.1–99.9 and >100 μg/l, respectively, among study subjects with W/W genotype of GSTP1. However, non-significantly higher risks of developing carotid atherosclerosis were observed for cumulative arsenic exposures greater than 1.0 mg/l-year for various genotypes of GSTP1.

DISCUSSION

The atherogenic effects of ingestion inorganic arsenic through drinking water have been well documented. Serious studies carried out in Taiwan have reported the significant association between long-term exposure to arsenic in drinking water and the risk for the development of atherosclerotic vascular diseases such as ischemic heart disease, cerebrovascular disease, and peripheral vascular disease, showing a dose–response relationship (Wu et al., 1989; Chen, 1990; Chen et al., 1996; Tseng et al., 1996; Chiou et al., 1997). Current studies carried out in Poland, Chile, Mexico, Argentina, Japan and Xinjiang, China have also reported the significant association between the development of peripheral vascular disease and ingestion inorganic arsenic through drinking water (World Health Organization, 1981; Chen and Lin, 1994; Engel et al., 1994; Cebrian et al., 1994; Borgono et al., 1977; Hotta, 1989; Wang and Huang, 1994).

The leading cause of death and disability in developed countries is atherosclerosis. Multiple risk factors include diabetes mellitus and hypertension that predispose to the development of atherosclerosis. (Patel and Kent, 1998). Prevalence of atherosclerotic vascular diseases significantly increased among individuals with diabetes mellitus and hy-

pertension (Shantaram, 1999). Significant dose–response relationships between long-term exposure to arsenic through drinking well water and risks of hypertension and diabetes mellitus were reported in Taiwan (Lai et al., 1992; Chen et al., 1995; Tseng et al., 2000). The association between arsenic ingestion through drinking water and prevalence of hypertension and diabetes mellitus has also been found in Bangladesh (Rahman et al., 1998, 1999).

Significant age–sex-adjusted odds ratios of developing carotid atherosclerosis were 2.1 and 2.1 for arsenic concentrations of 50.1–99.9 and $\geq$100 μg/l, respectively, as compared with the reference group of $\leq$50 μg/l. A significant risk of developing carotid atherosclerosis has also been observed for cumulative arsenic exposures of 1.0–6.9 and $\geq$7.0 mg/l-year, respectively, as compared with $<$1.0 mg/l-year as the reference group. However, dose–response relationships between risk of developing carotid atherosclerosis and various arsenic exposure indices were not observed. In addition, a significant dose–response relationship between age and risk of carotid atherosclerosis was observed. It might be implied that age was a major risk factor of developing carotid atherosclerosis. Hypertension patients also had a significantly higher risk for the development of carotid atherosclerosis in our study. However, a higher risk of this disease was not observed for diabetes mellitus patients

Atherosclerosis is a pathological condition that underlies several important disorders including coronary artery disease, cerebrovascular disease, and diseases of the aorta and peripheral arterial circulation. A proposed concept that oxidized lipoprotein is involved in atherosclerosis lesion development was formulated from demonstrations that low-density lipoprotein can injure cells under certain conditions, which were later shown to be conditions that facilitated oxidation of the lipoprotein (Henriksen et al., 1979; Hessler et al., 1979, 1983; Morel et al., 1983). Some studies proposed evidence that arsenic induced oxidative stress in mammalian cells and that induced oxidative damage can result in genotoxicity (Nordenson and Beckman, 1991; Lee and Ho, 1994). A recent study has shown that reactive oxygen species are involved in the formation of deletion mutations of human chromosome 11 in a human–hamster hydride cell following arsenic treatment (Hei et al., 1998). Based on this evidence, arsenic might be involved in the formation of atherosclerosis because it could induce oxidative stress which would increase the level of oxidized lipoprotein.

Ingested inorganic arsenic is quickly absorbed into the bloodstream and transported mainly to the liver. In the liver, some inorganic arsenic is methylated into various organic arsenic metabolites, including MMA and DMA, which are less toxic then unmethylated inorganic arsenic and more efficiently excreted in urine (Vahter and Marafante, 1983, 1987, 1988; Vahter et al., 1986; Lei and Ma, 1998; Vahter, 2000). The methylation process may be recognized as a detoxification mechanism. Unmethylated arsenic and arsenite may be extensively bound to intracellular components and accumulate in tissues of the lung, kidney, hair and nails (Vahter and Marafante, 1983, 1987; Georis et al., 1990). MMA^{III} has been reported recently as a toxic methylated metabolite of inorganic arsenic in urine (Zakharyan and Aposhian, 1999; Aposhian et al., 2000). The low methylation capability of arsenic, high relative percentage of unmethylated inorganic arsenic and MMA^{III}, might increase the body retention of arsenic and subsequently increase the risk of related diseases.

GST are a large family of phase II detoxification enzymes that catalyze the conjugation of reduced GSH to a wide spectrum of hydrophobic and electrophilic compounds. They play important roles in protection mechanisms against chemical carcinogenesis. Many studies have suggested that GSH might be involved in the initial reduction of arsenate to arsenite and the subsequent oxidative methylation. GSH is a necessary enzyme for arsenic methylation, perhaps through the formation of arsenite that is the preferred arsenic form for methylation, or through conjugation with arsenic (Buchet and Lauwerys, 1985, 1987; Thompson, 1993; Zakharyan and Aposhian, 1999). In this study, a significantly high risk of developing carotid atherosclerosis was observed among study subjects with W/M or

M/M genotypes of GSTP1, showing an odds ratio of 2.0. Humans with null genotypes of GSTM1, T1, and P1 have been considered to be high risk groups for carotid atherosclerosis due to their GSH deficiency. However, there were non-significant differences in risks for carotid atherosclerosis between study subjects with various genetic polymorphisms of GSTM1, T1, and P1 with the same exposure levels to arsenic. It might be that there are many genes of the GST family and each genetic polymorphism site in each GST gene needs to be examined. In order to evaluate the synergistic effects of genes with arsenic exposure on the atherogenesis of arsenic, more genes need to be examined in future studies.

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Chronic Inorganic Arsenic Exposure Alters Heme Metabolism in Humans

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ABSTRACT

Arsenic (As) exposure causes oxidative damage to the liver, resulting in numerous biochemical changes, including the impairment of heme biosynthesis and degradative pathway. This study evaluates the profiles of urinary porphyrin excretion and their relation with porphobilinogen deaminase (PBG-D), uroporphyrinogen decarboxylase (URO-D), and uroporphyrinogen III synthetase (UROIII-S) enzymatic activities from heme biosynthesis pathway in individuals chronically exposed to As via drinking water in northern Mexico. In addition we evaluated the hepatic function of these subjects. We studied 51 individuals from 3 villages exposed to different As concentrations via drinking water. Nazareno (0.014 mg As/l), Santa Ana (0.1 mg As/l) and Benito Juarez (0.3 mg As/l). The more evident alterations in heme metabolism observed in the highly exposed individuals were: (1) small but significant increases in PBG-D and URO-D activities in peripheral blood erythrocytes; (2) excretion of excess porphyrins in the urine (porphyrinuria), mainly due to coproporphyrin III (COPRO-III) and uroporphyrin III (URO-III). Comparatively with individuals exposed to low As levels, the high As exposed individuals showed a significant increase in the serum activities of alkaline phosphatase (ALP) and bilirubin concentrations, the latter is a heme degradation product, suggesting an impaired hepatic clearance of conjugated bilirubin (cholestasis). In conclusion, chronic As exposure causes qualitative as well as quantitative alterations in human heme metabolism, and causes cholestasis; however, the severity of the effects appears to depend on magnitude, pattern, and duration of As exposure.

Keywords: arsenic; arsenic biomarker; chronic exposure; heme; porphyrin metabolism; bilirubin

INTRODUCTION

Chronic exposure of humans to inorganic arsenic (iAs) has been associated with many adverse human health effects, including cardiovascular, hepatic and renal diseases in addition to cancer in kidney, liver, lung, urinary bladder and skin (ATSDR, 1993; García-Vargas and Cebrian, 1996) Also it has been observed that arsenic (As) exposure causes impairment of the heme biosynthesis and degradative pathway (NRC, 1999). Heme biosynthesis is an essential pathway that occurs in all nucleated mammalian cells. Biosynthesis of heme involves eight enzymes (Fig. 1), five of which produce intermediate molecules that are collectively called porphyrinogens. Studies in rodents subchronically exposed to iAs have shown a substantial increase in the urinary excretion of porphyrins. Porphynuria associated with As exposure consisted of an increase of uroporphyrin (URO) or coproporphyrin (COPRO) in urine (Woods and Fowler, 1977; Martinez et al., 1983; Woods and Southern, 1989; García-Vargas et al., 1995). Exposure to gallium arsenide or indium arsenide in rodents also causes qualitative as well as quantitative alterations in urinary porphyrins (Goering et al., 1988; Conner et al., 1995). The porphynuria in humans chronically exposed to As is similar to that produced by As in animal models. Telolahy et al. (1993) reported an increase of urinary COPRO in smelter workers exposed to arsenic trioxide; García-Vargas et al. (1994) reported an exposure-dependent increase of URO excretion and significant reductions in COPRO III. Here the COPRO/URO ratio in urine was decreased. To evaluate the possible use of urinary excretion of porphyrins as well as enzymes involved in heme pathways, we have evaluated the urinary porphyrin profiles in groups with different histories of As exposure. Because hepatic function is a metabolic process functionally associated with heme biosynthetic pathway, we have also evaluated some indicators of liver function in the same subjects.

METHODOLOGY

Group Selection

Participants for this study were residents, between 15 and 60 years old, from rural areas located at the center of northern Mexico. The individuals were selected from three villages with different histories of As exposure via drinking water. Seventeen individuals (9 men and 8 women) were selected from village of Santa Ana, Coahuila, where they had been exposed for more than 35 years to 0.4 mg As/l in its drinking water. However, due to changes in the village's water supply, the As content has been reduced to an average concentration of 0.1 mg As/l during the 3 years before this study. The same numbers of individuals (10 men and 7 women) were selected from the village of Benito Juarez, Coahuila, that had 0.3 mg As/l in its drinking water. The reference group consisted of 17 individuals (5 men and 12 women) from the village of Nazareno, Durango, that had 0.014 mg As/l in its drinking water. The predominant form of As (<92%) in the drinking water of the villages studied was pentavalent inorganic As. All participants signed an informed consent form. After obtaining consent, each subject completed an exposure assessment questionnaire. This questionnaire requested demographic and occupational information, medical history, and length of residence time in their present home. All subjects estimated their drug, alcohol, or tobacco usage. Individuals who had been exposed to pesticides, alcohol, or porphyrinogenic drugs in the previous 3 months before the study began were not included in the study. In addition, individuals exposed to alcohol in the last 5 days or who displayed clinical porphyria due to chronic alcoholism were excluded from study.

Time-weighted As exposure values (TWE) were estimated for each participant based on the history of consumption of well water and their As concentrations (mg/years).

The biological samples (blood and urine) were obtained from each individual. The blood samples were collected by venipuncture, in tubes without heparin and were centri-

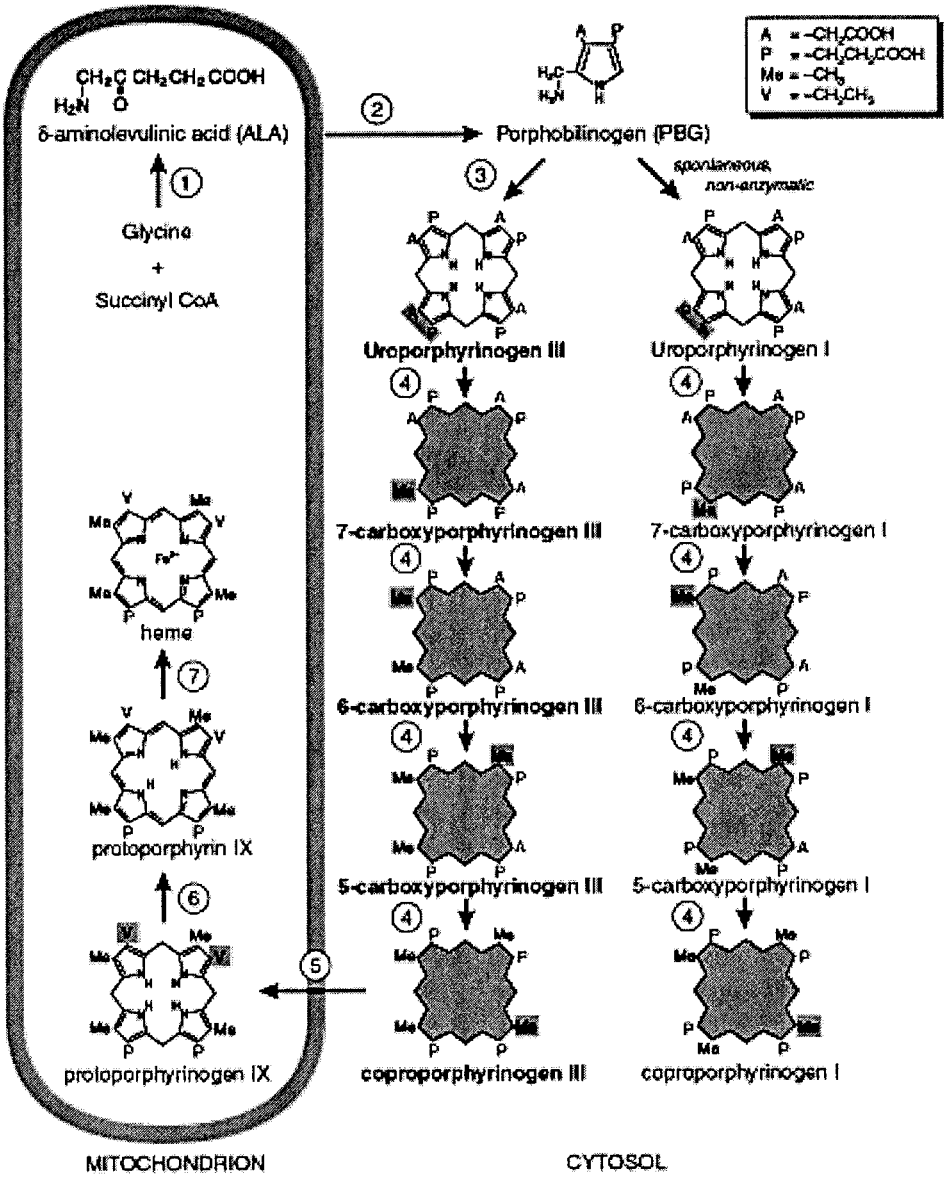

Fig. 1. Heme biosynthesis pathway. Steps are catalyzed by δ-aminolevulinate (ALA) synthetase, ALA dehydratase, porphobilogen deaminase (PBG-D) and uroporphyrinogen III cosynthetase (URO-III-S), two enzymes that work in concert, uroporphyrinogen decarboxylase (URO-D), coproporphyrinogen oxidase (COPRO-OX), protoporphyrinogen oxidase, ferrochelatase (heme synthetase). Figure reproduced with permission of MetaMetrix Clinical Laboratory.

fuged at 2000 *g* for 15 min. Serum and erythrocytes were separated and frozen at −20ºC until usage. First void urine samples were collected and stored in plastic bottles at −20ºC, protected from light until analyses of porphyrins and arsenic were performed.

Porphyrin Analysis

Urinary porphyrins were analyzed by HPLC, according to García-Vargas et al., 1994. The HPLC system was previously described (Hernández-Zavala et al., 1999). The accuracy of porphyrin determination was (mean ± s.d of five samples) 94 ± 3% for uroporphyrin and 111 ± 1.9% for coproporphyrin.

Enzyme Assays

The activities of porphobilinogen dehydratase (PBG-D) and uroporphyrinogen III cosynthase (URO III-S) were assayed simultaneously by the method Wright and Lim (1983). URO was eluted with 15% acetonitrile in 1 mol/l, ammonium acetate buffer pH = 5.16 and the flow rate was 1 ml/min.

The uroporphyrinogen decarboxylase (URO-D) activity was assayed essentially according to Lim et al. (1986). COPRO was eluted with 30% acetonitrile in 1 mol/l ammonium acetate buffer, pH = 5.16 and the flow rate was 1 ml/min. The enzyme activities were expressed as nmol of porphyrin formed per hour per ml of erythrocyte. All the assays were done in duplicate.

The activities of hepatic function indicators were determined by previously described ultraviolet and colorimetric methods (Hernández-Zavala et al., 1998). For quality control purposes, the reference material serum (QUALITROL HS-N Batch No. 411) was analyzed for all serum parameters at the same time as serum samples. We obtained an accuracy of 96–104% and a variation coefficient of 1.3–4.0% ($n = 8$).

Arsenic Analysis

Urine samples were assayed by hydride generation-atomic absorption spectroscopy (HGAAS) according to Crecelius et al. (1986). As species (inorganic As, monomethyl As, dimethyl As) were reduced to their corresponding hydrides and then detected using a Perkin Elmer 3100 AAS.

Quality control for TAs (iAs + MMA + DMA) included the analysis of freeze-dried urine standard reference material for toxic metals (SRM 2670) concurrently with urine samples from individuals. The certified concentration of the standard was 0.480 mg As/l. We obtained 0.488 mg As/l (range 0.447–0.522). The analytical accuracy was 93–109% and coefficient of variation was 1 to 9% ($n = 9$).

Creatinine in Urine

Creatinine was measured by an automated colorimetric method using a Vitalab Eclipse Merck spectrophotometer according to the recommendation of the German Society of Clinical Chemistry. Porphyrins and As concentrations in urine were corrected for creatinine excretion.

Statistical Procedures

Data analyses were carried out using the SAS® statistical program package (SAS Institute Inc., 1994). Comparisons between porphyrins concentrations and enzyme activities from As-exposed and control groups were made using the Mann–Whitney test. To assess the differences in hepatic function parameters and the urinary levels of As species between the groups of participants, Duncan's multiple range test was used. Pearson's correlation was used to study relationships between porphyrins concentrations, enzyme activities including parameters of hepatic function and urine As concentrations.

RESULTS

The data of Table 1 show that the highest urinary As concentrations were found in samples from Benito Juarez, which were 5- and 20-fold higher than those from Santa Ana

TABLE 1

Urinary arsenic concentration (iAs + MMA + DMA) and time-weighted arsenic exposure (mean ± s.d) of individuals from northern Mexico

Village	*n*	Arsenic in urine (mg/l)	TWE (mg/year)
Nazareno	17	0.088 ± 0.027	0.48 ± 0.28
Santa Ana	17	0.398 ± 0.258 *	13.80 ± 5.84 *
Benito Juarez	17	2.058 ± 0.833 *,**	3.36 ± 1.12 *,**

* $P < 0.05$ vs Nazareno; ** $P < 0.05$ vs Santa Ana, Duncan's test.

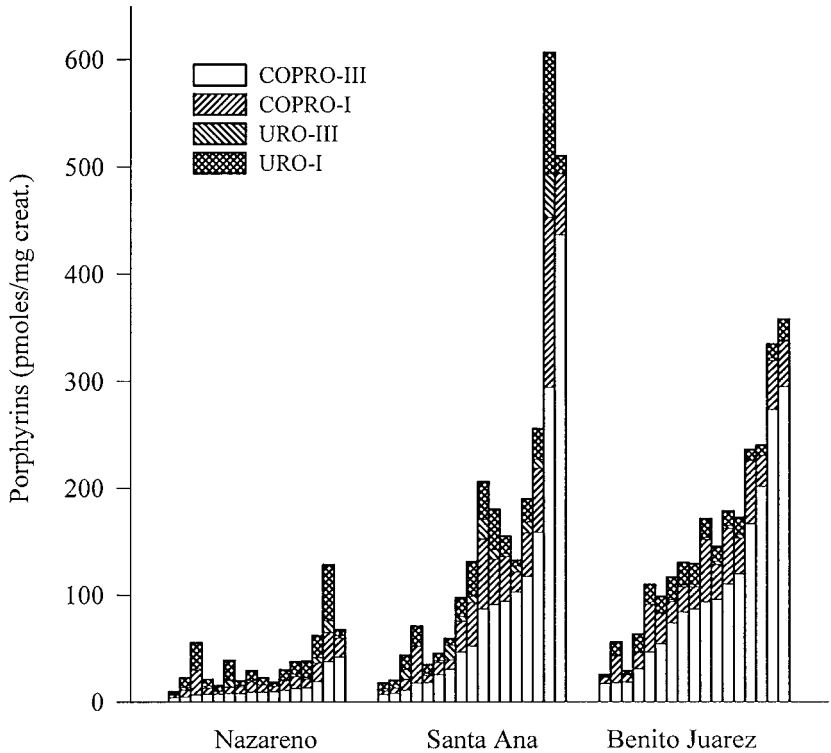

Fig. 2. Urinary porphyrins in chronic arsenic exposed subjects from northern Mexico.

or Nazareno, respectively. Mean As concentrations in urine from reference individuals (Nazareno) were below the proposed biomonitoring action level (0.1 mg As/l) (ATSDR, 1993). Regarding the chronic As exposure value of TWE (Table 1), Santa Ana was 4- and 29-fold higher than Benito Juarez or Nazareno, respectively.

Fig. 2 shows the profile of urinary porphyrins of each individual from the three villages. The levels of urinary porphyrins in exposed groups, Santa Ana and Benito Juarez, were significantly higher than in the low exposure group, Nazareno. Exposed people from Santa Ana, where the TWE was largest, presented higher concentrations (pmoles/mg

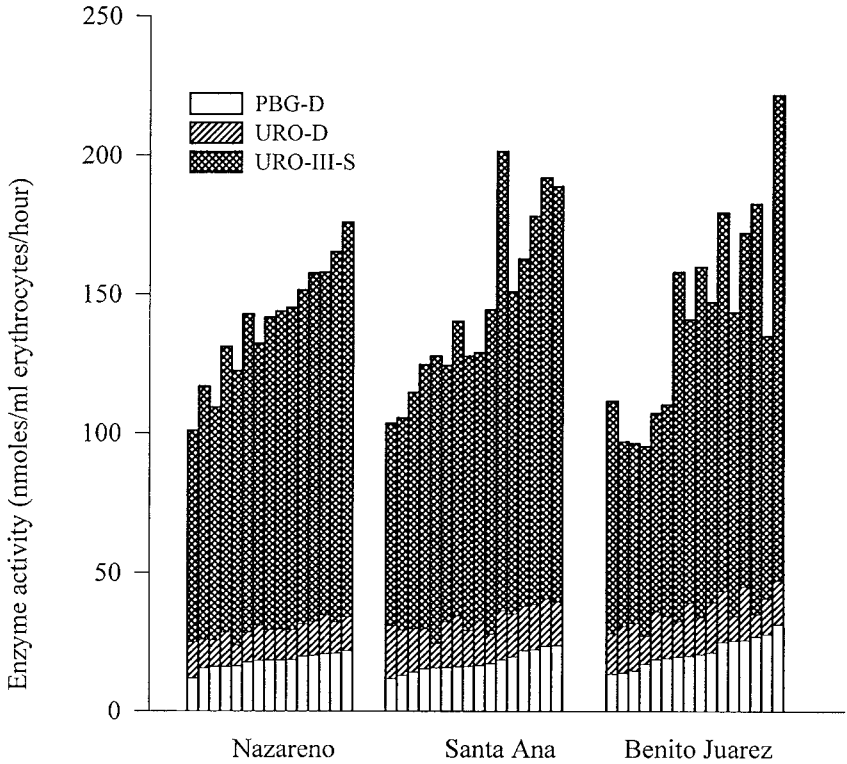

Fig. 3. Activities of enzymes porphobilogen deaminase (PBG-D), uroporphyrinogen decarboxylase (URO-D) and uroporphyrinogen decarboxylase (URO-D) in chronic arsenic exposed subjects from northern Mexico.

creatinine) of urinary URO-I (median 16.4 vs 14.1), URO-III (median 5.6 vs 2.1), and COPRO-I (median 34.1 vs 28.5) than exposed people from Benito Juarez. In contrast, the Santa Ana group presented lower concentrations of COPRO-III (52.6 vs 84.1 pmoles/mg creatinine) than did the Benito Juarez group.

Levels of urinary intermediates of porphyrins, seven-, six- and hepta-carboxylic porphyrins, from the Santa Ana group were higher than those of the Benito Juarez and Nazareno groups (data not shown). However, this difference was not statistically significant.

The individual activities of PBG-D, URO-D, and Uro III-S are shown in Fig. 3. Subjects from high As exposed groups, Santa Ana and Benito Juarez, had significant higher activities ($P < 0.05$) of URO-D and PBG-D than did subjects from the Nazareno group. Uro III-S activity was similar in all participants. We have found a small but significant direct relationship between PBG-D activity and the concentration of total porphyrins in urine ($r = 0.17$; $P < 0.05$), and between URO-D activity and COPRO in urine ($r = 0.29$; $P < 0.05$). Similarly, we found a significant relationship between URO-D activity and the urinary concentration of As ($r = 0.41$; $P < 0.05$); and between total porphyrins and As in urine ($r = 0.53$; $P < 0.05$). The direct relationships between enzyme activities of heme metabolism and the concentration of As in urine suggest that As exposure altered the activity of the hem synthetic pathway.

Both high As exposed groups, Santa Ana and Benito Juarez, showed significant in-

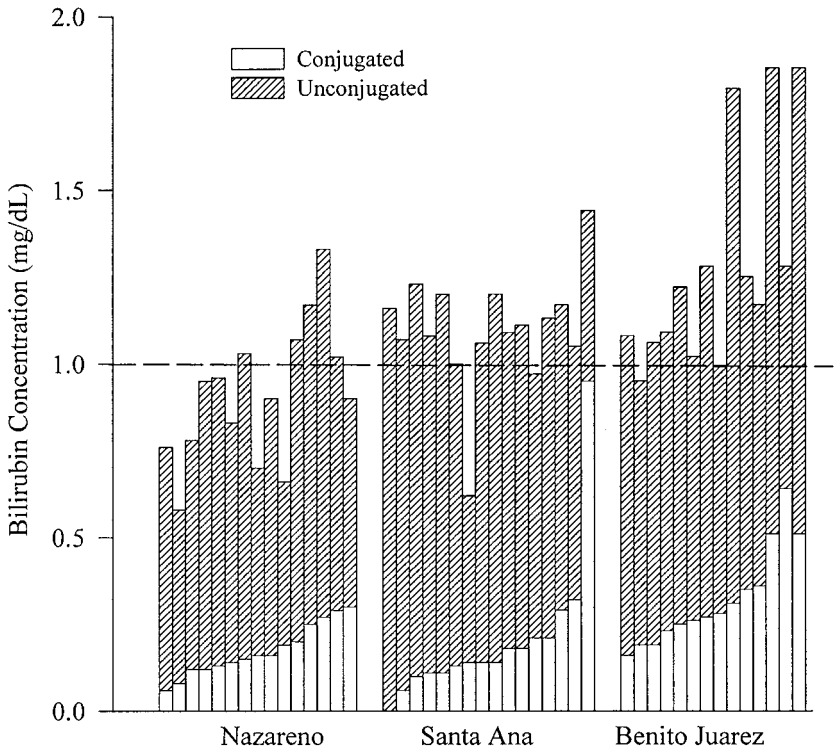

Fig. 4. Bilirubin concentration in serum from chronic arsenic exposed subjects from northern Mexico. Dashed line shows the normal value of total bilirubin in serum.

creases in the median ± s.d. values of alkaline phosphatase (ALP) activities in serum (230 ± 54 and 272 ± 89 UI/l, respectively) as compared with those of Nazareno (179 ± 43). Total bilirubin concentrations were also slightly but significantly elevated, 18 and 30% (mean 1.09 and 1.2 mg/dl) for Santa Ana and Benito Juarez, respectively, compared with Nazareno (mean 0.92 mg/dl); however, median value of conjugated bilirubin increased 76% in Benito Juarez. Individual values of bilirubin in each serum sample are shown in Fig. 4. No significant changes were observed in the other indicators of hepatic function (data not shown). Additionally, multivariate analysis including age, pesticides, alcohol and tobacco use, did not modify the above-mentioned results.

DISCUSSION

The main finding in this work was an increase in all measured metabolites of heme biosynthesis in humans exposed to As via drinking water. Thus, there were dose–response relationships between the As exposure indicators and total porphyrins, as well as each one of the intermediates (URO and COPRO) in heme biosynthesis. The levels of total URO (median: 14.9, range 3.8–70 nmol/l) and total COPRO (median 122.8, range 8.3–339.8 nmol/l) in urine of both high As exposed groups of this study were higher than those considered as normal. Woods et al. (1993) reported that levels of porphyrins in persons without porphyria or exposure to porphyrinogenic chemicals are typically characterized by moderate concentration (~1.5–9 nmol/l) of URO, low concentration (~0.05–5 nmol/l)

of seven-, six- and penta-carboxyl porphyrins, and relatively high concentrations (~15–45 nmol/l) of COPRO.

As exposure was evaluated using the total concentration of the metalloid in water, the total concentration of iAs and its metabolites in urine, and the TWE. All these measurements were consistent with historic knowledge of As exposure in each town.

The increases of both isomers I and III of urinary URO and COPRO, found in high As-exposed individuals were consistent with the increases observed in the activities of PBG-D and URO-D, enzymes that are involved in the decarboxylation of uroporphyrinogen to coproporphyrinogen, and also is consistent with the view that As exposure caused an overall stimulation of the heme biosynthesis pathway. The stimulation of heme biosynthesis can be explained as a feedback response resulting from the induction of heme oxygenase (HO-1). As a consequence, increased degradation of HO-1 can deplete cellular heme pools and could trigger a rise in heme biosynthesis. Exposure to As has been shown to induce oxidative stress in liver (Flora, 1999; Santra et al., 2000) and induction of HO-1 activity (Cebrian et al., 1988; Menzel et al., 1998; Kitchin et al., 1999). This view is supported with the observation of significant increases in the concentration of conjugated, non-conjugated, and total bilirubins in serum of the subjects of the current study. The increased concentration of bilirubin is an expected consequence of induction in HO-1 activity. Albores et al. (1989) reported significant increases in liver secretion of bilirubin in rats acutely treated to As; bilirubin was produced from recently synthesized heme. This finding was closely related with HO-1 induction and the doses of As administered to the animals.

The results reported in this work show a different response with those published with similar populations in the same region (García-Vargas et al., 1991, 1994) where the main findings in high exposed subjects were a discrete but significant increase of URO in urine accompanied by reductions in COPRO III and decreases in the COPRO/URO ratio. In other words, there were no increases in the urinary excretion of total porphyrins, but there were qualitative changes in the relationship among intermediate porphyrins and their isomers. Previous studies (García-Vargas et al., 1991, 1994) showed significant decrease in COPRO/URO ratio in high As-exposed individuals with respect to controls. The latter study also reported a reduction in the COPRO III/COPRO I ratio. Although, As concentrations in water from Benito Juarez and Santa Ana villages were significantly higher than those reported in Nazareno, it should be emphasized that the concentration of As in water from highly exposed towns have been variable since 1993, when drinking water with lower As content became available. Thus, before 1993, the As concentrations were consistently above 0.4 mg/l (García-Vargas et al., 1994); however, the current average of As concentration in water is 0.1 mg/l. On the other hand, Benito Juarez is usually offered with drinking water from different sources, although all of them with high As concentrations. The higher levels of As in urine from the Benito Juarez group were probably due to substantially higher aggregate exposure to iAs. Foods (soup and beans) commonly consumed in this rural area (Valentine et al., 1994) are cooked with contaminated water and contain high concentrations of As (Del Razo et al., 2001). The intake of As from food was not determined for participants in the present study; however, it was probably high. These variations in As exposures can explain the differences with previous studies cited above. Further studies are needed to identify the sources of the differences found in earlier research. Better characterization of the magnitude, pattern, and duration of exposure are critical for a better understanding of the relationships that underlie the alterations of heme metabolism during chronic exposure to As.

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Arsenic Exposure and Health Effects IV
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Mutations in C57Bl/6J and Metallothionein Knock-out Mice Induced by Chronic Exposure of Sodium Arsenate in Drinking Water

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ABSTRACT

Inorganic arsenic compounds have been classified as Group 1 carcinogens by IARC. Despite its strong human epidemiological evidence of arsenic carcinogenicity, the evidence for its carcinogenicity in animals is limited. As part of the mechanistic study of arsenic carcinogenesis, we investigated the competitive alkylation of DNA using arsenic alone and in presence of fotemustine, a known potent alkylation agent. Our results support that sodium arsenite [As(III)], sodium arsenate [As(V)], monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), even at orders of magnitude higher than physiological concentrations, do not alkylate DNA in vitro. In an in-vivo experiment, mice were given up to 20 mg/kg of arsenic in the form of sodium arsenite by the intraperitoneal route, no DNA adducts were detected in the liver of mice 0–48 h after dosing using the ^{32}P post-labelling technique. We exposed virgin C57Bl/6J and metallothionein knock-out (MT^{-1}) female mice to drinking water containing 500 μg As/l for over 2 years; high incidences of tumours were developed in a variety of tissues, particularly in lung, intestinal tract and liver. Twenty-six tissues of various organs systems from 12 C57Bl/6J and 11 MT^{-1} tumour-bearing mice were selected for detection of mutation in exons 5 and 7 of the *p53* gene using PCR, SSCP and cycle sequencing techniques. 9/12 (75.0%) of C57Bl/6J and 8/11 (72.7%) of MT^{-} mice developed one or multiple mutations in exon 5 of the *p53* gene. Mutation in codon 163 of exon 5 was observed from 9/12 (75%) and 10/14 (71.4%) of the tissues tested in C57Bl/6J and MT^{-} mice, respectively. Other mutations of lower frequencies were also observed in codons 148, 149 of exon 5 and codon 233 of exon 7. To our knowledge, this is the first point mutation observed resulting from arsenic exposure in an in-vivo animal model. In conclusion, our studies suggest that point mutation in the *p53* gene is not likely due to the direct binding reaction of arsenic and DNA because arsenic per se does not appear to alkylate the DNA nor does it form DNA adducts. Mutation patterns in other genes induced by chronic exposure of arsenic require further investigation.

Keywords: arsenic; p53; DNA damage; mutation; carcinogenesis; SSCP; cycle sequencing; drinking water

INTRODUCTION

Arsenic is a ubiquitous element in the environment, which is produced commercially by reduction of arsenic trioxide with charcoal. Arsenic trioxide is produced as a by-product of metal smelting operations. It is present in flue dust from the roasting of ores, especially those produced in copper smelting. Because of its prevalence in nature combined with its toxicity, the potential for its contamination of water, air and soil from both geological and anthropogenic sources is a significant environmental concern (WHO, 1981).

Inorganic arsenic compounds have been classified as a human carcinogen based primarily on epidemiological evidence (IARC, 1987; ATSDR, 2000). As an environmental toxicant and carcinogen it imposes significant health impacts on both humans and animals. It has been estimated that over 30 million people in India and Bangladesh are currently at risk as a result of drinking arsenic-contaminated waters (Das et al., 1995; Dhar et al., 1997). Many already have developed skin cancer and internal cancers involving other organs. Arsenicosis is also prevalent in certain areas of Guizhou, Shanxi, Xinjiang and Inner Mongolia of PR China and in Taiwan (Chen et al., 1999).

The acute and sub-acute effects of arsenic involve organ systems including the respiratory, gastrointestinal, cardiovascular, neurological, haematological, immunological, reproductive, dermal, hepatic and renal systems (ATSDR, 2000). Elevated arsenic concentrations in drinking water have been implicated in the increased incidence of prostate cancer in Victoria, Australia (Hinwood et al., 1999) and in Utah, USA (Lewis et al., 1999). There is also a correlation between oral exposure to arsenic and incidence of cancer of the skin, liver and other organ systems (Tseng, 1977; Chen et al., 1986).

Recently Ng et al. (1999) demonstrated for the first time the induction of tumours in C57Bl/6J and MT^- mice chronically exposed to arsenic (500 μg As/l) in their drinking water for over 2 years. The incidence of tumours in various organ systems of 90 C57B/6J and 140 MT^- mice, respectively, was lung (17.8%, 7.1%), gastrointestinal tract (14.4, 12.9), liver (7.8, 5.0), spleen (3.3, 0.7), reproductive (3.3, 5.0), skin (3.3, 1.4), bone (2.2, 0) and eye (1.1, 0). Examples of gross pathology are shown in Fig. 1. No tumours were observed in the corresponding control groups of 60 animals.

The mechanism of action of arsenic as a carcinogen is not well understood but there are some data to suggest that it causes large deletions (Hei et al., 1998) or methylation changes (Mass and Wang, 1997; Zhao et al., 1997) that could affect gene expression. It has also been suggested that the mechanism of inorganic arsenic carcinogenicity involves inhibition of DNA repair mechanisms (Li and Rossman, 1989) and/or oxidative damage of DNA (Yamanaka and Okada, 1994; Matsui et al., 1999).

Although heavy metals such as nickel (Duguid and Bloomfield, 1995; Duguid et al., 1995) and cadmium (Prakash et al., 2000) are known to bind to DNA directly, there are no such reports supporting arsenic interactions with DNA. There is however in-vivo evidence for the presence of oxidative DNA damage in skin neoplasms in arsenic-induced Bowen's disease (Matsui et al., 1999). In addition, significant levels of mutations in *p53* gene in the bladder tumours of Taiwanese have been observed in areas of Taiwan where high arsenic levels were found in the artesian well water (Shibata et al., 1994). Alterations in the *p53* expression have also been observed in arsenic-related basal cell carcinoma (Boonchai et al., 2000a,b).

As part of the study on the mechanism of arsenic carcinogenesis, we set out to investigate whether point mutations exist in tissues of tumorous mice chronically exposed to arsenic-spiked drinking water. The direct and indirect interactions between arsenic and DNA in both in-vivo and in-vitro systems were also further investigated.

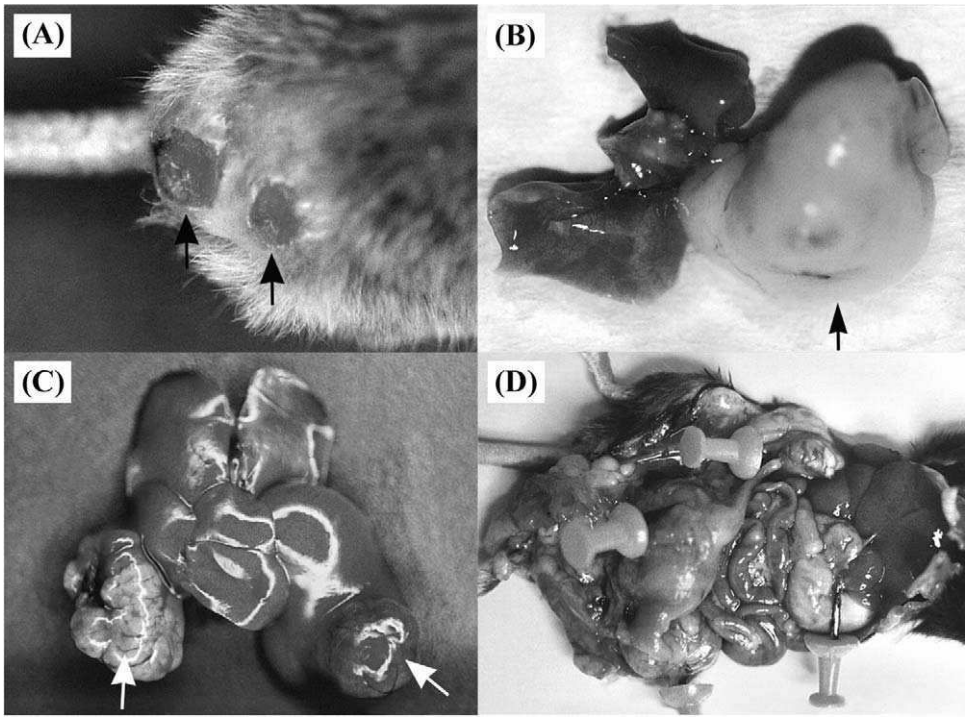

Fig. 1. Multiple tumours of the skin (A), liver (B), lung (C) and gastrointestinal tract (D) observed in mice exposed to 500 μg As/l via drinking water for over 2 years. Tumours are indicated by arrows and pins. This is an animal model for the study of arsenic carcinogenicity established for the first time at the National Research Centre for Environmental Toxicology (NRCET).

MATERIALS AND METHODS

Reagents

All laboratory reagents were of analytical grade and purchased from Ajax Chemicals (Australia). Dimethylarsinic acid (DMA) was purchased from Sigma–Aldrich Chemicals (Castle Hill, NSW) and monomethylarsonic acid (MMA) from ChemService (West Chester, PA, via Alltech, Australia). All biochemical were purchased from Sigma–Aldrich unless otherwise specified. Primers Taq DNA polymerase was from Genework, Australia; Polyacrylamide gel, silver stain (161-2449), APS and Temed from Bio-Rad; cycle sequencing kit from Gibco BRL; Milipur ConCert Rapid PCR purification system from Life Technology; PNK from Boehringer Mannheim; X-ray film (XARS), film-developer and fixer from Kodak. High purity water (18 MΩ-cm type I water) was obtained by passing demineralised water through a Milli-Q water purification system (Millipore, Bedford, MA).

In-vitro DNA Alkylation Pattern Studies

A 375 base pair *Eco*RI to *Bam*HI fragment of plasmid pBR322 DNA was 3′ end labelled at the *Eco*RI site using Klenow fragment and [α-^{32}P]dATP (3000 Ci/mmol) according to a published procedure (Prakash et al., 1990). The fragment was isolated on a 4% non-denaturing polyacrylamide gel. A partial sequence of the pBR322 DNA used in this work is shown below:

^{31} 5′GCTTTAATGC GGTAGTTTAT CACAGTTAAA TTGCTAACGC AGTCAGGCAC

$^{3'}$ CGAAATTACG CCATCAAATA GTGTCAATTT AACGATTGCG TCAGTCCGTG
^{81} CGTGTATGAA ATCTAACAAT GCGCTCATCG TCATCCTCGG CACCGTCACC
GCACATACTT TAGATTGTTA CGCGAGTAGC AGTAGGAGCC GTGGCAGTGG
^{131} CTGGATGCTG-$^{3'}$
GACCTACGAC-$^{5'}$

For studies involving As-induced alkali-labile DNA lesions, labelled DNA (at 10,000 cpm) was incubated with each 10 μM, 100 μM or 1 mM of sodium arsenate(V), sodium arsenite(III), MMA and DMA at 37ºC in 10 mM triethanolamine–EDTA buffer (pH 6.1, 6.8 or 7.6) for 60 min. In order to detect reversible binding of As to DNA, the ability of the arsenic compounds to inhibit the alkylation of DNA by fotemustine was probed by coincubating fotemustine (125 μM or 250 μM) treated DNA with each arsenic compound. See figure legends for details. All reaction mixtures were then precipitated in ethanol and treated with 1 M piperidine at 90ºC for 10 min. This causes cleavage of DNA at the alkali-labile sites (Prakash et al., 1990). The solvent was removed by lyophilisation before the samples were dissolved in a sequencing dye (95% formamide). The samples and sequencing lanes corresponding to guanine and purine were electrophoresed on a 6% denaturing polyacrylamide gel at 50ºC till the xylene cyanol dye front had migrated 25 cm. The gel was dried and exposed to Kodak XAR-5 X-ray film which was then developed.

In-vivo Experimentation

All animal experimentation protocols were approved by the University of Queensland/Queensland Health Scientific Services AEEC (Animal Experimental Ethics Committee). Virgin female C57Bl/6J black mice of 4 weeks old, metallothionein knock-out transgenic (MT^-) mice of 4 weeks old, Wistar rats of 6 weeks old and Quackenbush mice of 6 weeks old were purchased from the Central Animal Breeding House, The University of Queensland (Pinjara Hill, Brisbane). MT^- mice were originally derived from C57BL/6J through genetic manipulation and supplied to the University of Queensland by Murdoch University (Australia). All animals were fed on a standard commercial rodent diet (Norco Pty Ltd., Brisbane) and given water *ad libitum* via stainless steel sip tubes attached to polycarbonate water bottles; they were allowed to acclimatise for 1 week before experimentation. The animals were kept in polypropylene rodent cages with stainless steel wire mesh tops (Disposable Products, S.A., Australia). The animal caring facility situated at the National Research Centre For Environmental Toxicology was operated at a controlled temperature set at 21–23ºC, 12–13 filtered air changes per hour with a 12/12 light and dark cycle and year-round relative humidity of 60 ± 10%.

Chronic Exposure

90 C57Bl/6J and 140 MT^- mice were given drinking water containing 500 μg As/l *ad libitum* for up to 26 months. 60 control animals of each strain received normal water with arsenic < 0.1 μg/l. At necropsy, blood, liver and kidney were collected for arsenic analysis. Lung, liver, kidney, GI tract and other organs were collected and immediately fixed in 10% buffered neutral formalin (BNF) for histopathological examinations (Ng et al., 1999). Extra samples were collected for molecular biology studies. All specimens were stored at −80ºC until analysis. 26 tissues from tumour-bearing mice were used for the mutation studies (see Table 1 for details).

In Vivo DNA Adducts

Pairs of Quackenbush mice were given an intra-peritoneal injection of sodium arsenite at 0, 10, 20 mg As/kg. Animals receiving the highest dose (toxic dose) were sacrificed at 2.5 h. Animals receiving 10 mg/kg dose were sacrificed at 2.5 h, 24 h and 48 h time intervals. At necropsy, the liver of each mouse was excised and stored under liquid nitrogen temperature until ^{32}P post-labelling analysis. A negative control using DNA isolated from liver of

TABLE 1

Mutations in exons 5 and 7 of *p*53 gene from various tissues of tumourous C57Bl/6J and metallothionein knock-out (MT^-) mice induced by chronic exposure of sodium arsenate in drinking water (500 μg As/L) over two years.

Sample No.	Tumour tissue	Tissue type	Exon 5 codon, type	Exon 7 codon, type
1 (controls)	None	Liver	None	None
C57BL/6J				
4	GI tract	Liver	163, CCG→CXG	None
5	GI tract	Liver	None	None
6	Lipoma	Liver	163, CCG→CXG	None
7	Lung and GI tract	Liver	163, CCG→CXG	None
8	Lung and liver	Liver	163, CCG→CXG	None
9	Liver	Liver	163, CCG→CXG	None
14	GI tract	Tumour	148, TGT→GGT	None
			163, CCG→CXG	
16	Lung and lipoma	Liver	None	None
17	Lipoma	Tumour	148, TGT→GGT	None
			163, CCG→CXG	
18	Liver	Tumour	163, CCG→CXG	None
20	Lung	Lung	None	None
21	Lung and liver	Lung	163, CCG→CXG	None
MT−				
2	GI tract	Liver	163, CCG→CXG	None
3	Lung and GI tract	Liver	None	None
10	Lung, liver and GI tract	Liver	148, TGT→GGT	None
			163, CCG→CXG	
11	Liver	Liver	None	None
12	GI tract	GI tract	147, CCC→CCX	None
			163, CCG→CXG	
13	Lung and GI tract	Tumour	163, CCG→CXG	None
15	Lung	Liver	None	None
19	Lung	Lung	163, CCG→CXG	None
22	Liver	Liver	163, CCG→CXG	233, ATG→ACG
23 *	Lung, liver and ovary	Liver	149, GCA→TCA	None
24 *	Lung, liver and ovary	Liv. tumour	163, CCG→CXG	None
25 *	Lung, liver and ovary	Lung	163, CCG→CXG	233, ATG→ACG
26 **	Skin, liver and bladder	Skin	149, GCA→TCA	None
			163, CCG→CXG	None
27 **	Skin, liver and bladder	Lung	163, CCG→CXG	None

Where controls = several negative control livers were assayed to confirmed published DNA sequence for normal mice; GI tract = gastrointestinal tract; X = T, A, G.
* 23–25, same mouse.
** 26 and 27, same mouse.

animals received no arsenic and a positive control using benzo-(A)-pyrene (BP) were run for each batch of assay. The ^{32}P post-labelling method for measurement of DNA adducts was based on a nuclease P1 enhancement procedure (Gupta, 1993).

DNA Extraction

DNA extraction was carried out as described elsewhere (Moore, 1994). Briefly, about 250 mg of tissue from each sample was ground using an electrical homogeniser and was incubated in 3 ml digestion buffer [100 mM NaCl, 10 mM Tris HCl, pH 8, 25 mM EDTA, pH 8, 0.5% sodium dodecyl sulphate, 0.1 mg/ml fresh proteinase K (Boehringer Mannheim)]

at 50ºC for 18 h in tightly capped tubes on a shaker. The samples were then extracted with an equal volume of phenol–chloroform–isoamylalcohol mixture (25 : 24 : 1) and centrifuged for 10 min at 17,000 *g*. DNA was precipitated in cold ethanol and recovered by centrifugation at 17,000 *g* for 2 min. DNA quantification was carried out in Tris-EDTA (TE) (pH 8.6) buffer using Cary UV spectrophotometer. Samples having A_{260}/A_{280} ratios between 1.8 and 2.0 were used for DNA analysis.

Polymerase Chain Reaction

Exons 5 and 7 of *p53* genes were amplified from the DNA of these tissues using the following primers: exon 7 sense and antisense primers CAG GTC ACC TGT AGT GAG GTA GGG and TGG AAC AGA AAC AGG CAG AAG CTG G, respectively; exon 5 sense and antisense primers ACA CCT GAT CGT TAC TCG GCT TGT C and GGA GGA GCC AGG CCT AAG AGC AA, respectively (Goodrow et al., 1992; Trukhanova et al., 1998). These primers overlap intron/exon regions in order to exclude pseudogene amplification. The samples were amplified using thermal cycling in a total volume of 50 μl containing 0.5 μg of DNA extracted from the tissues. The PCR buffer containing 67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2$ SO_4, 0.2 mg/ml gelatin, 0.45% Triton X-100, 150 μM of each dNTP and 50 pmol of each primer, 1.5 mM $MgCl_2$ and 2 units of *Taq* polymerase (GeneWork, Australia) was used. The PCR conditions for exon 7 were (95ºC × 10 min) followed by (90ºC × 1 min, 55ºC × 30 s and 72ºC × 30 s) × 10, followed by extra [(90ºC × 1 min, 57ºC × 30 s, 72ºC × 30 s) × 10] and [(90ºC × 1 min, 55ºC × 30 s, 72ºC × 30 s) × 10]. The final incubation was 5 min at 72ºC. The PCR conditions for *p53* gene exon 5 were the same as for exon 7. Confirmation of PCR products was carried out using 10 μl aliquot of each sample on a 1.5% agarose gel. The PCR products were filtered through a polysulphonate membrane (Millipur ConCert Rapid PCR purification system), to remove excess nucleotides and primers prior to SSCP and cycle sequencing analyses.

Single-Stranded Conformational Polymorphism (SSCP)

SSCP was carried out on a 10% polyacrylamide mini-gel (Hoefer Scientific) and allowed to run for 2.5 h at room temperature. After electrophoresis the gels were visualised using the Bio-Rad silver stain (Goldman and Merril, 1982).

Cycle Sequencing

Sequencing of purified exons 5 and 7 for *p53* fragments was carried out using dsDNA Cycle Sequencing System (Gibco BRL). Reaction mixtures containing 1 pmol of 5′ end-labelled sequencing primer (sense or antisense primers shown above) and 15–50 pmol of DNA template (from control and treated samples) were amplified using 1.25 units of *Taq* polymerase and termination mix. The cycling conditions were (95ºC × 1 min) × 1, 95ºC × 30 s, 58ºC × 30 s, 70ºC × 60 s) × 20 followed by 10 cycles of a denaturation step at 95ºC for 30 s and an extension step at 70ºC for 60 s. The samples were then dissolved in a sequencing dye, denatured by heating at 90ºC for 5 min followed by cooling on ice. Electrophoresis was carried out at 45ºC on a 6% polyacrylamide denaturing gel. The gel was subsequently dried and exposed to Kodak XAR5 X-ray film.

RESULTS

Fig. 2 shows in-vitro DNA alkylation patterns obtained using competitive DNA-binding assays. It is evident that As(V) (lane b), As(III) (lane c), MMA (lane g) and DMA (lane h) do not induce alkali-labile damage to DNA directly. In order to determine if arsenic is involved in reversible binding to DNA we carried out a competitive binding assay (Shelton et al., 1996) using fotemustine, a well-characterised major groove alkylating agent (Hayes, 1997). Again only marginal changes to fotemustine alkylation intensities were observed

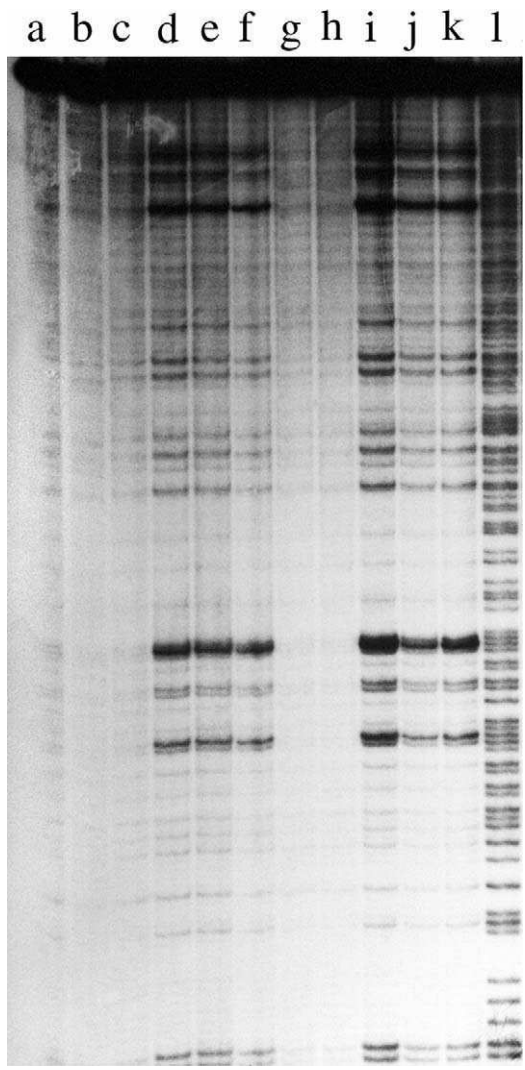

Fig. 2. An example of DNA alkylation patterns of in-vitro studies using As(V), As(III), MMA, DMA with or without FM. Where: lane a = normal DNA; b = DNA + 1 mM As(V); c = DNA + 1 mM As(III); d = DNA + 250 μM FM; e = DNA + 1 mM As(V) + 250 μM FM; f = DNA + 1 mM As(III) + 250 μM FM; g = DNA + 1 mM MMA; h = DNA + 1 mM DMA; i = DNA + 250 μM FM; j = DNA + 1 mM MMA + 250 μM FM; k = DNA + 1 mM DMA + 250 μM FM; l = Pu lane.

even at concentrations (up to 1 mM) well above normal exposure levels (compare lanes d–f and i–k). These results suggest that arsenic compounds do not interact with DNA directly.

No DNA adducts were observed in any of the liver samples from mice treated with a single lethal or sub-lethal I/P dose of sodium arsenite up to 48 h after dosing (results not shown here). The results were validated against negative control and positive BaP-DNA adduct control.

Fig. 3 shows the SSCP patterns of DNA (exon 5, *p53*) from representative control and affected animals. It is evident that the sample DNA in lane 2 has two bands not observed in the control DNA or from other dosed animals (lanes 3–8). We were unable to assign the extra SSCP bands observed for lane 2 to any particular mutation of exon 5

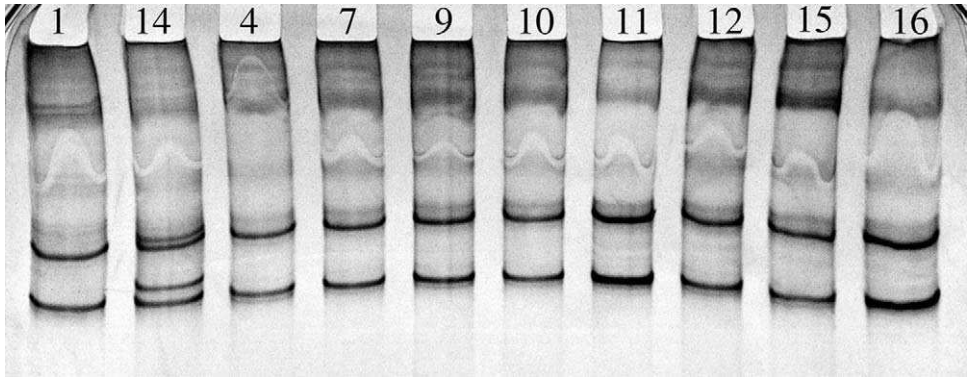

Fig. 3. SSCP patterns of DNA (exon 5, *p53*) from C57Bl/6J and metallothionein knock-out mice where No. 1 is a liver sample from a normal control mouse. Other lanes representing a selection of various tissues from arsenic-treated mice (see Table 1 for details). Only sample 14 from the GI tract lymphoma is showing mutant bands.

based on cycle sequencing information. Further sequencing of the extra band of lane 2 did not provide additional information. Sequence analysis using cycle sequencing technique provided mutation information in some animals thus far tested. The full details of mutation results are summarised in Table 1. The major hotspot for mutations was in codon 163 which carried multiple mutations in both MT^- (72.7%) and C57BL mice (75%). Other mutations in codons 147, 148 and 149 were also found in exon 5 while 2 MT^- animals carried mutations in codon 233 of exon 7. Examples of sequences obtained from exon 5 and exon 7 of DNA samples from selected animals are shown in Figs. 4 and 5.

DISCUSSION

It has been suggested that inorganic arsenic affects DNA repair mechanisms and is thus implicated as a carcinogen. This is supported by human epidemiological evidence of the carcinogenic effects of inorganic arsenic from inhalation exposure (Enterline et al., 1987; Ferreccio et al., 1995). There is also a correlation between oral exposure to arsenic and incidence of cancer of the skin, liver and other organ systems (Tseng, 1977; Chen et al., 1986). On the basis of these epidemiological studies, inorganic arsenic compounds have been classified as human carcinogens (IARC, 1987).

Previous studies of arsenic genotoxic effects afforded mixed results. It has been sugges-

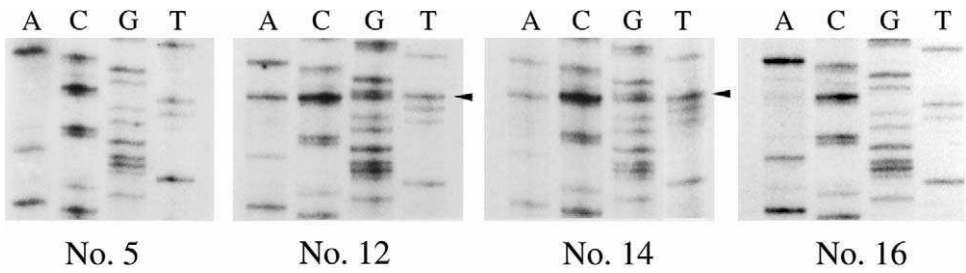

Fig. 4. Cycle-sequencing gels of DNA (exon 5, *p53*) from tissues of arsenic-treated mice. Arrows indicate point mutations locations. Tissues 5 and 16 show the normal sequencing in that region whilst tissues 12 and 14 show CCG → CXG in codon 163. Details of point mutations are listed in Table 1.

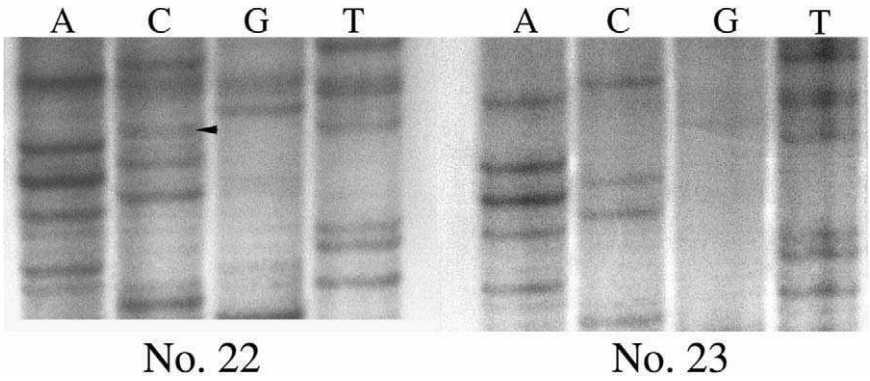

Fig. 5. Cycle-sequencing gels of DNA (exon 7, *p53*) from tissues (No. 22 and 23) of arsenictreated metallothionein mice. Sample No. 22 shows ATG → ACG mutation in codon 233 (arrow heads indicate mutation region).

ted that ingestion of arsenic may cause chromosomal aberrations and/or sister chromatid exchange but the published results are not conclusive (ATSDR, 2000). It was also thought that inorganic arsenicals are either inactive or weak mutagens (Jacobson-Kram and Montalbano, 1985).

Our ^{32}P results confirm that arsenite does not interact with DNA directly in vivo. In addition, in-vitro DNA alkylation assays suggest that arsenate, arsenite, their metabolites MMA or DMA do not alkylate DNA even at higher than realistic physiological concentrations. These findings are not entirely unexpected as arsenate and arsenite at physiologic pH are present as anionic forms in solution. Negatively charged species do not bind with DNA which is also negatively charged. T.G. Rossman (unpubl. data, 1999) failed to induce any DNA strand breaks or alkali-labile sites using arsenite incubated with supercoiled plasmid DNA. Rossman (1999) suggested that inorganic arsenic neither forms DNA adducts, nor does it induce the DNA–protein cross-links characteristic of another oxyanion, chromate (Zhitkovitch and Costa, 1992).

Using a mouse model we have recently found that C57BL/6J and MT^- (metallothionein knock-out) mice exposed to drinking water containing 500 μg As/l for over 2 years developed tumours in lung, intestinal tract, liver, spleen, reproductive, skin, bone and eye (Ng et al., 1999). The highest tumour incidences were found in lungs (17.8%, 7.1%), intestinal tract (14.4%, 12.9%) and liver (7.8%, 5.0%) of arsenic-treated C57Bl/6J and MT^- mice while 3.3% and 1.4% of these two strains of mice had skin cancers (Fig. 1). We chose to compare the effect of chronic arsenic exposure in these two strains because it is believed that metallothionein (MT) may play a role in the protective mechanism against arsenic toxicity via sequestration and elimination (Kreppel et al., 1993). We would expect to see a higher incidence of tumours in the MT^- mice. However, our results suggest that MT does not appear to offer any significant protection in mice exposed chronically to arsenic.

It is also worthy of note that arsenic seems to induce tumours in multiple organ systems in our mouse model particularly involving the lung, liver and skin. This is similar to epidemiological observations in humans from arsenic-endemic areas. Chemicals causing tumours in multiple organs have been reported previously. For example, ptaquiloside, a natural toxin isolated from bracken fern when administered to the rat orally induced tumours in several organ systems including the bladder, liver and lung (Hirono et al., 1987; Smith, 1997). Similarly, non-specific target organ carcinogenicity of arsenic is evident in our study.

A recent arsenic study conducted in Taiwan (Hsueh et al., 1995) reported that the prevalence of skin cancer was 6.1%, and that there was a significant dose–response rela-

tionship between skin cancer prevalence and duration of residence in the endemic area. Our mice were not exposed to sunlight; in addition, interspecies differences between humans and rodents may explain the lower skin cancer incidence observed in laboratory animals.

This present study was carried out using the animal tissues obtained from our earlier tumour studies described above. The results showed that the tumour suppressor gene *p53* was mutated in most of the 26 tissues of treated mice. The overall mutation rates were found to be 75.0% (9/12) and 72.7% (8/11) in C57Bl/6J and MT$^-$ mice, respectively. Mutation hotspot was found in codon 163 of exon 5 (in 75% and 71.4% of the tissues tested). Interestingly, both species showed similar frequency (71.4% vs 57.1%) of mutations in 5/7 and 4/7 liver samples. Some tissues have multiple mutations including samples 12, 14, 17 and 26. Other mutations were found in codons 147, 148 of exon 5 in both species while mutation in codon 233 of exon 7 occurred only in MT$^-$ animals. In this regard it is interesting to note that MT$^-$ cells show enhanced apoptosis and elevated levels of *p53* expression (Zhou et al., 1997). It should be noted that all mutations reported here were observed using the cycle sequencing technique. SSCP proved to be a less sensitive technique in identifying mutations. During the SSCP screening of mutants in the 26 tissues from treated animals, only sample 14 returned a mutant band when compared to normal control and other treated animals. Unfortunately, we have not been able to identify this 'mutant band' (lane 2 of Fig. 3). This band does not correspond to mutations detected in codon 148 or 163 as these mutations were also observed in samples 7 and 10.

For comparison with other DNA-damaging agents, Trukhanova et al. (1998) found that CBA mice treated with 1,2-dimethylhydrazine developed uterine sarcoma and harboured mutations in codons 148, 241 and 242. However, CD-1 mouse liver and lung tumours induced by 34 different alkylating agents did not show *p53* mutations in any of the exons 5–8 (Goodrow et al., 1992). To our knowledge, this is the first report of in-vivo mutations caused by metals other than those by radioactive isotopes.

The mechanism of mutations caused by arsenic exposure alone is not clear yet. One possible route is the production of oxidative radicals and subsequent DNA damage. Recently, monomethylarsonous acid (MMA(III)) was detected in urine of humans exposed to inorganic arsenic (Aposhian et al., 2000). MMA(III) has been shown to be an order of magnitude more toxic to Chang human hepatocytes in vitro than its parent compound arsenite (Petrick et al., 2000). Whether MMA(III) can damage DNA directly in vivo leading to its carcinogenic effects remains unknown. Our results confirm that arsenic can cause multiple tumours in mice chronically exposed to inorganic arsenic resulting in multiple mutations in various tissues. It would appear that arsenic is a mutagenic metalloid contrary to previous claims based on mainly in-vitro data. This mouse model should serve as a useful tool for further studies of arsenic carcinogenesis. Mutations in other exons of *p53* gene, *Ras* and *hOG* genes in a larger pool of samples are being investigated.

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Arsenic Exposure and Health Effects IV
W.R. Chappell, C.O. Abernathy and R.L. Calderon (Editors)
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Arsenite Exposure Causes Both Hypomethylation and Hypermethylation in Human Cell Lines in Culture at Low Concentrations

Cathy Zhong, Liangjun Wang, Marc J. Mass

ABSTRACT

We and others have hypothesized that a mechanism of arsenic carcinogenesis could involve alteration of DNA methylation since this process utilizes a methyltransferase and consumes S-adenosylmethionine (SAM) as the methyl donor. We analyzed differentially methylated regions of genomic DNA from human lung A549 cells and three human kidney cell lines (UOK cell lines) after arsenite treatment using methylation-sensitive arbitrarily primed PCR (MS-APPCR). Relative to untreated controls, six DNA fragments identified by MS-APPCR were hypermethylated and two were hypomethylated in DNA from these lung and kidney cell lines. Two of the DNA fragments contained repeat sequences of mammalian-apparent long terminal repeat retrotransposons (MaLRs), and five were identified to be promoter-like sequences. DNA methylation alterations were manifested at low nanomolar concentrations in some cell lines. The mRNA for DNA methyltransferase (MTase) increased in cells exposed to arsenite over a 2-week period. Our results show the occurrence of both hypermethylation and hypomethylation of DNA after exposure to arsenite. The results support the existence of a state of methylation imbalance (hypo- and hypermethylation) that could conceivably disrupt gene expression in arsenic exposed cells in vitro.

Keywords: arsenic; DNA methylation; hypermethylation; hypomethylation

INTRODUCTION

Arsenic is well documented in several studies in exposed human populations to be a carcinogen. It is associated with increased risk of skin, liver, kidney, bladder, and prostate cancers when ingested in drinking water, and is associated prominently with lung cancer via inhalation (USEPA, 1988; Chen et al., 1992; Smith et al., 1992; Nriagu, 1994; Chiou et al., 1995; Tsuda et al., 1995).

Alteration of DNA methylation can be a potential mutagenic event (Jones, 1996; Yang et al., 1996), it can modify gene expression (Gonzalez-Zulueta et al., 1995; Herman et al., 1995; Merlo et al., 1995) and can have developmental consequences (Li et al., 1992; Feil and Khosla, 1999). Changes in DNA methylation are among the most common of changes associated with carcinogenesis (Jones, 1996).

Arsenic is not recognized to be a classical genotoxic agent. Neither arsenite nor arsenate are electrophilic and neither appear to damage DNA directly. Inorganic arsenic does not induce significant point mutation (Rossman et al., 1980), but it can inhibit certain DNA repair processes, though not necessarily directly (Li and Rossman, 1989; Hu et al., 1998). It induces sister-chromatid exchange (Jha et al., 1992) gene amplification (Lee et al., 1988), aneuploidy (Gurr et al., 1993), and chromosome aberrations (Nakamuro and Sayato, 1981; Ramirez et al., 1997). Among the arsenicals tested in the mouse lymphoma LY5178Y TK +/− mutagenesis system, arsenite is the most potent mutagen followed by arsenate, monomethylarsonic acid (MMA(V)), and dimethylarsinic acid (DMA(V)) (Moore et al., 1997). Inorganic arsenic will malignantly transform Syrian hamster embryo cells (Lee et al., 1985), and an immortalized rat hepatocyte line exposed to arsenite gave rise to undifferentiated tumors when injected into nude mice (Zhao et al., 1997). Most recently (Mass et al., 2001) the trivalent methylated arsenicals have been shown to exhibit extraordinary genotoxic activity.

Arsenic is apparently biotransformed to methylated forms by a methyltransferase (MTase), utilizing S-adenosylmethionine (SAM) as a methyl donor although non-enzymatic methylation using methylcobalamine has been reported (Zakharyan and Aposhian, 1999). At present, a specific arsenite MTase has not been cloned or sequenced but enzymatic MTase activities have been fractionated from several sources (Zakharyan et al., 1996; Healy et al., 1998). Because DNA MTases also require SAM as a methyl donor, it has been postulated that carcinogenesis by arsenic could be mediated through changes in DNA methylation as a result of interference with a common set of pathways that encompass DNA damage/repair, cell cycle and differentiation by suppression of transcription of the appropriate genes or by inappropriate activation (Mass, 1992; Mass and Wang, 1997; Schroeder and Mass, 1997; Zhao et al., 1997).

The hypothesis that a mechanism of arsenic carcinogenesis might involve alteration of DNA methylation has been investigated in our laboratory; we showed in 1995 at the Second International Symposium on Arsenic and Health Effects (SEGH2) that exposure of human lung A549 cells to arsenite and arsenate over a 2-week period was associated with hypermethylation of a fragment of the *p53* promoter region containing major transcription start sites (Mass and Wang, 1996; Mass and Wang, 1997). We later showed that cytosine methylation in a portion of the human *p53* promoter region (from −199 to +142) can down-regulate transcription in a reporter plasmid construct (Schroeder and Mass, 1997). It is still unclear what the mechanism is for alteration of DNA methylation by arsenite since one laboratory has reported that arsenite exposure can induce global hypomethylation in cultured rodent cells and one laboratory has reported gene-specific hypermethylation in human cells.

In order to reconcile some of these observations we sought to explore the effects of arsenite in human cell lines where a gene (*VHL*; the tumor suppressor gene associated with von Hippel–Lindau Syndrome) was already known to be altered by DNA methylation,

and asked the question: what is the effect of arsenite exposure on methylation of this gene? We chose *VHL* because the expression of the VHL tumor suppressor gene, unlike *p53*, is well documented to be responsive to methylation of the 5′ control regions of *VHL* (Herman et al., 1994) and alterations expression of the *VHL* gene are strongly implicated in the genesis of human clear cell carcinoma of the kidney. The methylation status of the *VHL* gene in three human kidney cancer cell lines (UOK lines), two of which are unmethylated in the promoter/exon 1 region of *VHL* gene was examined. However, no arsenic-associated alterations of methylation of the promoter region of *VHL* were found in any of the UOK cell lines. For more detailed analysis we then utilized a methylation-sensitive variant of the technique arbitrarily primed methylation-specific PCR (MS-APPCR; Gonzalgo et al., 1997) to identify differentially methylated sequences of genomic DNA in the UOK cell lines and in A549 cells after exposure of cells to sodium arsenite in vitro. Both hypermethylation and hypomethylation were observed in cells treated with arsenite. We conclude that arsenite could produce a general state of DNA methylation imbalance that could explain the plethora of effects seen in arsenite treated cells, both activating and inactivating important cellular functions.

MATERIALS AND METHODS

Cell Culture, Cytotoxicity Assay and Sodium Arsenite Treatment

Human kidney cell lines UOK123, UOK109 and UOK121 (Anglard et al., 1992) were kindly provided by Dr. Robert Worrell (NCI, Bethesda, MD). The promoter/exon 1 region of the *VHL* gene is unmethylated in UOK123 and UOK109, but methylated in UOK121. All UOK cell lines were cultured in DMEM (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (Gibco BRL), 100 units/ml penicillin and 100 μg/ml streptomycin at 37ºC with 5% CO_2 . The A549 human lung cell line obtained from the American Type Culture Collection (Rockville, MD) was cultured as described previously (Mass and Wang, 1997). The culture medium was replaced twice weekly for all studies.

UOK cells were seeded in 6-well plates (35 mm wells) at 800 cells/well and exposed to sodium arsenite 24 h later after cells had attached. Cells were stained with 10% Giemsa and colonies were counted after growing in the presence of sodium arsenite for 10 days. All determinations were performed in triplicate.

Cells were exposed to sodium arsenite as described by Mass and Wang (Mass and Wang, 1997). For UOK cells, sodium arsenite was used at concentrations that yielded IC_{30}, IC_{50} and IC_{80}. For A549, IC_{20}, IC_{50} and IC_{80} were used (Mass and Wang, 1997). In brief, 20,000 cells from each cell line were seeded in one 75-cm^2 culture flask. Sodium arsenite was added 24 h later, and replaced twice a week in this series of treatments. This summarizes the growth protocol: when grown to 75% confluence, the cells were removed from the plastic support by trypsinization and split equally into three 75-cm^2 culture flasks. After the cells reached 75% confluence in the three 75-cm^2 culture flasks, the cells were divided into three aliquots and seeded into three 150-cm^2 culture flasks. The cells were collected for analysis when they were 75% confluent in 150-cm^2 flasks. For A549 cells this passage regimen required approximately 2 weeks. For the UOK cell lines this passage level was achieved in about 4 weeks because these cells grow more slowly than A549 cells.

Genomic DNA Isolation and Restriction Enzyme Digestion

Total genomic DNA was isolated using conventional methods with proteinase K and phenol/chloroform extraction. Before restriction enzyme digestion, genomic DNA was dialyzed at 4ºC overnight. Five micrograms of total genomic DNA were digested with 50 units *Rsa*I alone, with 50 units of *Rsa*I and 100 units of *Hpa*II, or with 50 units *Rsa*I and 100 units *Msp*I (Boehringer Mannheim, Indianapolis, IN) at 37ºC overnight.

Methylation-Sensitive AP-PCR

20-mer primers. PCR reactions of 25 μl in volume contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM primer (or 0.6 μM each primer if the two-primer combination was used; MGE2: 5′-AACCCTCACCCTAACCGGCC-3′; MGF2: 5′-AACCCTCACCCTAACCCG CG-3′; MLG2 5′-AACCCTCACCCTAACCCCGG-3′), 200 ng of digested DNA, 2 μCi α-^{33}P dATP and 1 unit Taq polymerase. The PCR protocol was initial denaturation at 94ºC for 5 min,; 5 cycles 94ºC for 30 s, low stringency annealing at 40ºC for 60 s, and 72ºC for 90 s, then 30 cycles of 94ºC for 15 s, 55ºC for 15 s, 72ºC for 60 s, followed by 4 min at 72ºC.

12-mer primers. PCR reactions of 25 μl in volume contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM primer (or 0.3 μM each primer if the two-primer combination was used; OP-JHind 12: 5′-AGCTTGTTCATG-3′, OP-NHind 12: 5′-AGCTTCTCCCTC-3′, OP-RHind 12: 5′-AGCTTGCGG TGA-3′), 200 ng digested DNA, 2 μCi α-^{33}P dATP and 1 unit Taq polymerase. The PCR protocol was initial denaturation of DNA at 94ºC for 5 min; then 35 cycles at 94ºC for 1 min, low stringency conditions of 40ºC for 1 min and 72ºC for 2 min, followed by 10 min at 72ºC. Each sample (from 12-mer or 20-mer primer-containing PCRs) was loaded and resolved on a 5% polyacrylamide sequencing gel of 0.4 mm in thickness under denaturing conditions (7 M urea). The gels were dried, and exposed to autoradiographic film.

Samples (from 12-mer or 20-mer primer-containing PCRs) were loaded and then resolved on a 5% polyacrylamide sequencing gel of 0.4 mm in thickness under denaturing conditions (7 M urea). The gels were dried, and exposed to autoradiographic film.

Isolation and Sequencing of DNA Fragments Generated by MS-APPCR

Candidate bands that were differentially methylated were excised from dried polyacrylamide gels and re-amplified by PCR using the same PCR reaction mix and amplification parameters as described for MS-APPCR above. PCR products were cloned into a plasmid vector using a TA cloning kit (Invitrogen, San Diego, CA). Positive clones were screened for an insert by PCR using M13 and T7 primers, and sequenced using the automated DNA Sequencer, ABI 377 (PE Applied Biosystems, Foster City, CA).

For homology searches, the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/) was used. Repetitive sequences were identified using the REPEATMASKER program of University of Washington (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). Potential promoter regions were predicted using the TSSG and TSSW programs of Baylor College of Medicine (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html) and the NNPP program of University of California at Berkeley (http://www.fruitfly.org/seq_tools/promoter.html).

Methylation Status of the Promoter/Exon 1 Region of VHL Gene in UOK Cell Lines

Total genomic DNA (5 μg) was digested with 100 units of *Hpa*II and *Msp*I (Boehringer Mannheim), respectively, at 37ºC overnight. Digested DNA then was amplified by PCR using primers VHLPF1 (5′-CAGTAACGAGTTGGCCTAGC-3′) and VHLE1R (5′-GTCGAAGTTGAGCCATACGG-3′). PCR reactions were 25 μl in volume and contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM of each primer, 100 ng of digested DNA and 1 unit Taq polymerase. The PCR protocol was 1 cycle of 94ºC for 5 min (before adding Taq polymerase), and 35 cycles of 94ºC for 1 min, 63ºC for 1 min, 72ºC for 2 min, followed by 1 cycle of 10 min at 72ºC.

Expression of DNA MTase mRNA in A549 Cells

RT-PCR. Total RNA was isolated from cells treated with sodium arsenite for 2 weeks using Trizol reagent (Gibco BRL), and the first strand of cDNA was synthesized using the Superscript Preamplification System (Gibco BRL). PCR was performed using primers MTase 1 (5′-GATAGA GATCAAGCTGCC-3′) and MTase 2 (5′-GAGTAGGTGCGCGAATTG-3′). The

50 μl PCR reactions contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM each primer, 5 μl cDNA mix, 10 μCi α-^{32}P dCTP and 1 unit Taq polymerase. The PCR protocol was 1 cycle of 94ºC for 4 min, and 30 cycles of 94ºC for 1 min, 48ºC for 2 min and 72ºC for 3 min, followed by 1 cycle of 5 min at 72ºC. A 25 μl aliquot of the reaction was loaded on an agarose gel. The band containing the radioactive amplified products was excised, and the ^{32}P content was quantitated by scintillation counting. For an internal control, the gene β-actin was amplified using primers β-actin1 (5′-TCGACAACGGCTCCGGCATG-3′) and β-actin2 (5′-CGTACATGGCTGGGGTGT-3′) under the same conditions except the annealing temperature was 55ºC. Assessment in changes in mRNA were analyzed statistically using one-way ANOVA with Tukey's modification.

RESULTS

Cytotoxicity of Sodium Arsenite to UOK Cell Lines

To establish the appropriate concentrations for exposure of UOK123, UOK109, and UOK121 to arsenite, the cytotoxicity of sodium arsenite was measured by a colony-formation efficiency assay. These three cell lines were very sensitive to sodium arsenite compared to other human cell lines (Mass and Wang, 1997; Salazar et al., 1997; Hamadeh et al., 1999). The IC_{30}, IC_{50} and IC_{80} were determined to be (a) 10, 20 and 50 nM for UOK123, (b) 7, 21 and 93 nM for UOK109, and (c) 9, 20 and 74 nM for UOK121. In subsequent studies, UOK123, UOK109 and UOK121 lines were exposed to sodium arsenite at IC_{30}, IC_{50} and IC_{80} For A549 cells, sodium arsenite at IC_{20} (80 nM), IC_{50} (400 nM)) and IC_{80} (2000 nM) (Mass and Wang, 1997) was used.

Methylation Pattern of the Promoter/Exon 1 of the VHL Gene

Primers VHLPF1 and VHLE1R were designed to amplify the promoter/exon 1 region of the *VHL* gene. In this region, there are 10 CCGG sequences (Latif et al., 1993). As expected, placental DNA, and DNA from UOK123 and UOK109 grown in the absence of sodium arsenite yielded 0.5 kb PCR products from uncut DNA, but not from DNA cut with *Hpa*II or *Msp*I. No PCR products were obtained from digested DNA of UOK123 and UOK109 treated with IC_{30}, IC_{50} and IC_{80} of sodium arsenite for one month, indicating that there was no hypermethylation detected by this technique in this region. For UOK121 which normally has a methylated *VHL* promoter/exon 1, PCR products were obtained from uncut DNA as well as DNA cut with *Hpa*II regardless of sodium arsenite treatment for one month, suggesting that there was no hypomethylation in *VHL* promoter/exon 1 associated with arsenite exposure that could be detected using this technique.

Identification of Differentially Methylated DNA Fragments Using Methylation-Sensitive AP-PCR

Methylation-sensitive AP-PCR was performed on digested DNA using a single or a combination of two primers under low-stringency conditions. Using this technique, we identified a total of eight differentially methylated DNA fragments, i.e., one hypermethylated DNA fragment in UOK123, four hypermethylated DNA fragments in UOK109, one hypomethylated DNA fragment in UOK121, and one hypermethylated fragment and one hypomethylated DNA fragment in A549 induced by sodium arsenite (Table 1).

Differentially methylated DNA fragments were excised from polyacrylamide gels, and amplified again to generate sufficient amount of pure DNA templates for cloning. All differentially methylated DNA fragments were cloned and sequenced (Table 2). DNA sequence analysis revealed that none of the eight DNA fragments had significant homology matches to known gene sequences in the GENBANK database after BLAST search. However, two MaLRs from one differentially hypermethylated fragment in UOK123 and one differentially hypomethylated fragment were identified from A549 cells by using the REPEATMASKER program (Table 1). To examine the presence of potential promoter re-

TABLE 1

Summary of characteristics of differentially methylated DNA sequences in human cell lines exposed to arsenite in culture identified by MS-APPCR

Identity	Cell line	Primer	Methylation status	Classification
Sequence 1	UOK109	OP-NHind 12	Relative hypermethylation	Promoter-like
Sequence 2	UOK109	OP-NHind 12	Relative hypermethylation	Promoter-like
Sequence 3	UOK109	OP-NHind 12	Relative hypermethylation	Promoter-like
Sequence 4	UOK109	OP-RHind 12	Relative hypermethylation	Promoter-like
Sequence 5	UOK121	MGE2 + MGF2	Relative hypomethylation	Promoter-like
Sequence 6	A549	MGF2	Relative hypermethylation	Unidentified
Sequence 7	A549	MGF2 + MGL2	Relative hypomethylation	MaLRs element
Sequence 8	UOK123	OP-JHind 12	Relative hypermethylation	MaLRs element

TABLE 2

DNA methyltransferase m-RNA expression in cell lines grown in the presence of sodium arsenite

Arsenite concentration (μM)	1 day	2 days	7 days	14 days
0.08	1.0[a]	1.1	1.3	1.5[b]
0.4	0.9	1.2	1.5[b]	2.6[b]
2.0	0.9	1.6[b]	2.0[b]	3.2[b]

[a] DNA methyltransferase m-RNA activity relative to untreated control.
[b] Significantly different than untreated control at same time period as assessed by one-way ANOVA using Tukey's modification.

gions in these DNA sequences, promoter prediction was performed using the TSSG, TSSW and NNPP computer programs. Five out of eight sequences were predicted as putative promoters (Table 1).

Expression of DNA MTase mRNA in A549 Cells

The mRNA level of DNA MTase was examined in A549 in cells exposed to 0.08. 0.4, and 2.0 μM arsenite for 24 h, 48 h, 1 day, and 2 days of exposure in culture. No increases in DNA MTase mRNA were noted in the first 24 h for any of the arsenite concentrations. After 48 h of exposure to arsenite at 2 μM, DNA MTase mRNA was about 1.6-fold greater than the untreated control ($P < 0.05$). After 1 week of exposure, DNA MTase mRNA was about 1.5-fold greater at the 0.4 μM concentration than untreated controls; at 2 μM the increase was 2.8-fold ($P < 0.05$ for both concentrations). After 2 weeks of exposure to 0.08, 0.4 and 2 μM arsenite, the increases in DNA MTase mRNA were approximately 1.5-, 2.6-, and 3.15-fold over untreated controls ($P < 0.05$) (Table 2).

DISCUSSION

Our laboratory reported previously that dose-dependent hypermethylation of a fragment of the *p53* promoter region in A549 cells was observed after approximately 2 weeks of exposure to arsenite at low micromolar concentrations. The concentration of arsenate required to achieve the same effect on methylation of the *p53* promoter seen with arsenite was 10–100 fold greater with arsenate (Mass and Wang, 1997). The linkage between alteration of methylation of the human *p53* promoter and expression of *p53* is not well established; however, there are now two publications from independent laboratories that have confirmed that regions of the *p53* promoter when methylated will inhibit the ex-

pression of a linked reporter gene (Schroeder and Mass, 1997; Pogribny et al., 2000). The effect of arsenite on *p53* expression in human cells in culture has been reported as varied. Arsenite was shown to be inhibitory to *p53* expression in human cell cultures (Hamadeh et al., 1999) and stimulatory (Salazar et al., 1997). More recently, Boonchai et al. (2000) reported that in human basal cell carcinomas associated with medicinal arsenic exposure, immunohistochemical expression of the p53 protein was significantly decreased compared with basal cell carcinomas associated with UV exposure (Boonchai et al., 2000).

To determine if arsenic exposure also induced alterations of DNA methylation in promoter regions of other tumor suppressor genes that might be important, we chose tumor suppressor gene *VHL*. The *VHL* gene is well-documented to be regulated by DNA methylation in clear cell kidney carcinomas (Herman et al., 1994), and there is some evidence of increases in kidney cancer in humans exposed to arsenic (Chen et al., 1992; Smith et al., 1992). Characterized cell line variants were available that had methylation alterations in the promoter/exon 1 region of the *VHL* gene and this made it possible for us to ask questions about the *VHL* methylation status after arsenic exposure to cells with *VHL* alleles that were initially unmethylated or methylated; this potentially gave us the possibility to observe hypermethylation or hypomethylation of the *VHL* promoter/exon 1. However, we did not observe the changes of methylation pattern in the promoter/exon 1 of the *VHL* gene after arsenite exposure for approximately 4 weeks in culture using *Hpa*II digestion followed by PCR amplification of the promoter/exon 1. We therefore used a more sensitive approach. MS-APPCR (Gonzalgo et al., 1997), can assess alterations of DNA methylation status at multiple regions in the entire genome. In the present study, we identified by MS-APPCR six differentially hypermethylated regions and two differentially hypomethylated regions of genomic DNA in three UOK cell lines, and in A594 cells after growth in the presence of sodium arsenite, relative to untreated controls. Our findings are consistent with previous report from our lab (Mass and Wang, 1997), i.e., arsenite can induce hypermethylation, but we found that it can also induce hypomethylation as has been reported elsewhere but in rodent cells (Zhao et al., 1997). In studies reported here hypermethylation is the predominant alteration observed. We also demonstrated that hypermethylation and hypomethylation could coexist in A549 cells grown in the presence of sodium arsenite. Similar findings (Gonzalgo et al., 1997; Liang et al., 1998) were reported with respect to tumor tissues and cell lines compared to normal tissues using the same technique of MS-APPCR, further emphasizing the importance employing techniques that can assess methylation status of multiple genomic loci.

Among eight differentially methylated DNA fragments associated with arsenic exposure, two contained MaLR elements, and five contained putative promoter sequences as identified by the TSSG, TSSW and NNPP computer programs. Changes of methylation patterns of repetitive regions are known to alter genome organization and chromatin structure; therefore, they could indirectly affect gene function (Godde et al., 1996; Kundu and Rao, 1999). We found that the promoter region of *p53* in A549 cells and four other putative promoter regions in UOK109 cells were hypermethylated, and one putative promoter region in the UOK121 cell line was hypomethylated after arsenite treatment. Hypermethylation and hypomethylation in promoter regions could directly affect gene expression (Herman et al., 1994; Gonzalez-Zulueta et al., 1995; Merlo et al., 1995). Changes of DNA methylation can presumably activate some genes and repress others in response to arsenite exposure.

The relationship between DNA MTase activity and cytosine methylation levels is not straightforward (Wu et al., 1993; MacLeod and Szyf, 1995; Kuerbitz and Baylin, 1996). Here, we observed higher mRNA levels of DNA MTase in A549 cells after sodium arsenite exposure relative to untreated controls, and the total genomic 5-methylcytosine content increased 36% (Mass and Wang, 1997).

Arsenic exposure can produce a variety of cellular effects. Arsenite is known to inhibit

the action of enzymes containing vicinal dithiols in their active site (Spiegel et al., 1976; Black, 1983), it inhibits oxidative phosphorylation (Guerrieri and Papa, 1982). Arsenite is an inhibitor of the selenomethyltransferase at the micromolar level (Hsieh and Ganther, 1977), and is also known to inhibit *Eco*RI DNA MTase in vitro (Tsao and Maki, 1991). There is evidence that arsenic can interfere with JNK phosphatase (Cavigelli et al., 1996) and other protein phosphatases (Huang et al., 1995), and cellular phosphorylation (Samet et al., 1998) and these might regulate the activity of DNA MTases and other enzymes that are involved in DNA methylation (DePaoli-Roach et al., 1986). Therefore, arsenite might interfere with DNA methylation in several pathways. The effect of arsenite may not be predictable depending on the stage of the cell cycle the cells are in at the time of treatment and the particular pathways that are active and susceptible to arsenite inhibition at the time of exposure. The consequence may be an *imbalance* of methyl group metabolism including DNA methylation.

The fact that unique methylation changes of both hyper- and hypomethylation have been observed at various loci in four human cell lines after exposure to sodium arsenite brings to light the possibility that the changes of DNA methylation patterns could be a random event, or it could be a selective event. It is known that arsenite can cause extensive chromosomal damage (Nakamuro and Sayato, 1981; Lee et al., 1988; Jha et al., 1992; Gurr et al., 1993; Ramirez et al., 1997) and inhibit DNA repair systems (Li and Rossman, 1989; Hu et al., 1998). If DNA damage cannot be repaired by DNA repair systems, such damaged cells may die because they can not replicate due to severe unrepaired DNA damage. However, cells with altered methylation patterns that do not sustain lethal DNA damage conceivably retain a selective advantage for survival and proliferation because they can still replicate though their methylation patterns are changed, hence propagating changes in gene expression. This might be a mechanism for development of a heritable resistance to arsenic toxicity that does not rely on direct DNA damage or mutation.

Although we have seen changes in methylation of the *p53* promoter in A549 cells in association with arsenic exposure, we were unable to see changes in the methylation pattern of the promoter of the *VHL* gene in the UOK cell lines by the simple enzyme restriction/PCR technique we used earlier (Mass and Wang, 1997). We are left with the possibility that arsenic generally (non-specifically) perturbs DNA methylation patterns since MS-APPCR showed both hyper- and hypomethylation relative to untreated controls, and this occurs at low frequency. It is possible that the methylation changes that give cells in culture a selective advantage for growth are the methylation changes that are likely to be seen, and these may differ depending on the origin of the cell lines and their growth requirements.

At this point in time there are no reports of the methylation status of promoter regions of genes examined from tumors associated with endemic arsenic exposure. These tumors may contain signature changes in gene structure, gene expression, or DNA methylation patterns that may be peculiar to arsenic induced cancers. It will remain important to examine DNA methylation changes in promoter regions of genes in tumors associated with arsenic exposure.

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(U.S. EPA). The views expressed in this paper are solely those of the authors and do not necessarily reflect views or policies of the U.S. EPA. Mention of trade names or commercial products does not constitute recommendation or endorsement for use.

APPENDIX

DNA Sequences isolated from human cell lines identified by MS-APPCR to undergo cytosine methylation changes after exposure to arsenite:

Sequence 1: 405 bp

5′-TCTTTCTTAAACACCCCCATACCGGCGACGGTATTATGCCTTCCGGCGCCCCTTA
GCTCTAAGTTACGTACCTCCATCGTGGAGGTTCGGTAGGGCTTCGGTCCTCTTAAGG
CTATGAAGATGAGGGGGTTCTGCTTAACTAGTTCTACAGTTAGGTGAGTTTAAGTTG
CGGGCGGAGGCGGTTGAGAGGACGTCGCCTCGAACCCACGTTCCCACTTTCGATTT
TAGCAGGTTTTTGGGACTTCCTTCAGTACCGATCGTCCCGAGCCCGCCGACGACGC
CGGCCAACGTTCGCTCTTGTCATTGGGTTATCTCTCGAGAGGTAGAGATCTCCGTCC
CCGCTATCGTCGCGCTTTTAAGCACGCGGTATAAGCGCTCGGTATACGGCAGATTA
CTAAACAAAAA-3′

Sequence 2: 330 bp

5′-TGAACCTCGTAAGACCAAGTCCTCTTTCTTCGGGACGCTCTATAGTATTCGGTAC
ATAGGCTCTTATATAAGTAAGCCTCTCGCGCGCCGCGGTAAGCGGCGCTAAGACCC
GGACGCCCGCGAAATCATAAAGATCGTAGGACCTGGTAACATTATCCGCGGGTAC
GCGCGCGTGCGCGCGCGCTCGCGAACGACTAAGCCGGAAACTCGGGATACCCTCC
CCGAACCACCCCGAGAATCTCGCCCGTAAGCAAAAGCGAGTCTCCAGGTGTAATC
GCTACCCTTCGATTGCGTCGCTAACGCGAGGGAGAAGTCAACTTACTATATCGG-3′

Sequence 3: 339 bp

5′-GTCACTCGTAGAAACATAGTCCAAATTTCTAATAAAACGTCCATTAAGCCCAAC
AAAAAGAAGACCTACCGAAGACCCTCCAAGCTAAGACGAAGAACTCAAACGCCG
CCCTACGAACACATTGCGGTAAGTAAGCGTGCTAATCAAACCCGAGCGGATAAGA
AGTCGCAACCAGGGAGATAAAGCTAGAAGGTATCCGTAATCTAGATTCGTCAAGT
TAGCACATCCGTAGCGGCGAGGGAGAAGTCAAGTTGAACCTTAGTATATCGGGTG
GGAGCCATCAAGCGGACTTGAGTCTGGCATTAAGTCCGGTGCAACTACGGCTACA
GTCGCCCTTCG-3′

Sequence 4: 316 bp

GACACTCGTAGAACTGGTCCAATCCGTGGCGACTACCCCATAAAGCACACGCACA
CTAAAATACTACTCCGCATATTCCAAACACACATAGCCCCCACCCGTTAACTGTAT
CCTAATAAAGTCGGGAGAAAAATCTGCCTTCCCCTCACATTACTTCTCCACCGCCCT
ACAGAGACAACGCCCCACTCTCACGAAGAAAACATCGATCCACCGCCCGACCGCC
CGCCCCGAGATTAAGCTCTGTCTCGCTGTTTAGGTCGGCGCGTAGCGGTGTGACTCT
GGTCTATTGTAAGTCAAGTGAACCTTAGTATATCGG

Sequence 5: 373 bp

TATTAGGGGGATAGAAATTATACGGCACGTCCAAATAAGGGAAGCCCAGTACAAG
AACCACCAATCACCCATAGGGAAGCCGATCAAGAGGGAATAAAAGAGAAACTTA
TAGAATACTTCAGAGCAAAGGGAGGCAATCAAGGGAAGGGACCTAGATTCAGGA
GAGGGCGCGACTAAAAGTATGTATCAGACGACGATGCTGATCGGGTCGCTTTAGG
ATAGAATCGAGGGTTAAGAATTATGCGTGAGGATGTCAATATAGCGAAGTCGGAA
GGTCGTCCGAGGAGCGTCGGAAAGTTGTGGGCCAGGCGAGGGCCAACTCGTAGAA
CCTGGTCAAGTTGAACCTTAGTATATCGGTGGTTGCCATCACCGG

Sequence 6: 508 bp

AACCCTCACCCTAACCCGCGGCCATTCTTGAAAGCATTAAGTTGCCCTACAGTGGG
CCACATCCCCTTCTTGAGAGGTACCAAAGAACTTGCTCCGCTCTCTAGTGATGGCCC
GCTGACCCCAGACAACAGCCTTTCTGAGACGATGGCTTTTATCAGTGGCTTGTCTCA
GGAAGCTGGAGCAATGTTAGGTAAAACTACACCCAATGCACCCAGAGAACTCAAC
AGAGCCAAGAATGTCAAAGGAAATCAGCCAGGATAGCTGCTGGTTACTGGTGTCA
CCACTCCCACCCACGCAAGAATGACTCTCAGCCACGGTGAAGACAAACTTTGTCTC
TTAAACTTTGACTCTCAGCCATGGTGAAGACAAACCTCACGTGAGCTTTTGCCAAG
CCCATTCAGCAGAATCTCGGGCCGCTTGGGACGCATCTTCTCCCTTGGGACACCGT
CTTCCCCTCAGTAGAGGCACCACTATACCAGCCTCTGTGCCCGGGGTTAGGGTGAG
GGTT

Sequence 7: 287 bp

TCTCCCTCATGAAGACAGACAGTAAATATCACAACGGACTCCCAACATCCCTTCTG
CCTTTCCTCTGCCAAACAGCAGCAGCACAATGTGAGGAATGTTCTTCTGCTCAAGG
AGTGGGTCAGCCTGGTTGATGGTAACCCTATTCTCCCTTGCCAATAGTTTTTTGAAG
AAAGGGGCTTATGACCCAATTCTGGACAATTACATGAAAGGAAATTTCTGCTGGAA
GCCAATAAAGGTATAGGAAACTAATTTATCTTTCCTGTTGGACAAGGAGGAGGGA
GAAGCTA

Sequence 8: 293 bp

AGCTTCTCCCTCCAGACACAAGTCTTTAAAATGCTGATCAGAGCATTGAAAACTGA
AACAGACACAATACCGACCTCCTGTGGTAGACTGACTCATGGTCCCACAGATGTCC
ATCCACATCCTAAACCCCAGAACCTATTACCTTACATGGTTCAAAGGATTTTGCAG
CTGTGACTAAGCTATGGACCTTAAAATGGAGAGATTACCCTAGATTATCCAGGTGG
GTTTGGTGTAATCACAAGGGTCCTCCTGGGAGGGAAGTGAGAGGATCAGAGTCAG
AAGAGGGAGAAGCT

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Arsenic Exposure and Health Effects IV
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Induction of Proliferative Lesions of the Uterus, Testes and Liver in Swiss Mice Given Repeated Injections of Sodium Arsenate

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ABSTRACT

Inorganic arsenic (As) is a human carcinogen but has not been unequivocally proven carcinogenic in rodents. For instance, one older study indicates that repeated i.v. injections of sodium arsenate might induce lymphomas in Swiss mice (58% incidence; Osswald and Goerttler, 1971), but it was considered inadequate for critical evaluation of carcinogenic potential largely because of issues in experimental design. Therefore, we studied repeated i.v. sodium arsenate injection and neoplastic response in male and female Swiss mice. Groups ($n = 25$) of mice received sodium arsenate (0.5 mg/kg, i.v.) or saline (control) once/week for 20 weeks and were observed for a total of 96 weeks when the study ended. Differences in survival and body weights were unremarkable. In females, arsenate induced marked increases in the incidence and severity of cystic hyperplasia of the uterus compared to controls. Arsenate also was associated with a rare adenocarcinoma of the uterus. Hyperplastic uterine epithelium from arsenate-exposed animals showed strong positive immunostaining for the proliferating cell nuclear antigen. There was also an upregulation of estrogen receptor immunoreactive protein in the early lesions of uterine luminal and glandular hyperplasia but a progressive decrease in its expression was seen in the severe hyperplastic or neoplastic epithelium. Arsenate-induced uterine hyperplastic lesions also showed a strong upregulation of cyclin D1, an estrogen-associated gene product essential for progression through the G1 phase of the cell cycle. In other tissues, arsenate increased testicular interstitial cell hyperplasia incidence and severity over control but without affecting the incidence of tubular degeneration. Arsenate also induced increases in hepatic proliferative lesions (foci of alteration + neoplasia), but only in females. Significant skin changes (incidence of hyperkeratotic lesions) also occurred in arsenate treated females. Thus, repeated arsenate exposure, though not outright tumorigenic in the present study, was associated with proliferative, preneoplastic lesions of the uterus, testes, and liver. Estrogen treatment has been associated with proliferative lesions and tumors of the uterus, female liver and testes in other studies, supporting a hypothesis that arsenate might somehow act through an estrogenic mode of action.

Keywords: arsenic; uterus; testes; liver; proliferative lesions; mice

INTRODUCTION

Inorganic arsenicals, in the form of arsenate (5+) and arsenite (3+), are common environmental toxicants and known human carcinogens (IARC, 1980, 1987; NRC, 1999). Inorganic arsenic compounds are multi-site carcinogens in humans, causing tumors of the lung, skin, and bladder (IARC, 1980, 1987; NRC, 1999). Other human studies indicate that the kidney, liver, and prostate may also be target sites of arsenic carcinogenesis (Lewis et al., 1999; NRC, 1999). Inorganic arsenic compounds can be carcinogenic in humans after occupational or environmental exposure (IARC, 1980, 1987; NRC, 1999), supporting the rationale for making arsenic a top priority hazardous substance to the population of the United States (Goering et al., 1999).

Although clearly a human carcinogen, inorganic arsenic has not been unequivocally demonstrated to be, by itself, carcinogenic in rodents (NRC, 1999). In fact, it is considered that chronic studies on the oral carcinogenicity of inorganic arsenicals with appropriate experimental design have provided consistently negative results (NRC, 1999). This creates the disconcerting possibility that humans, for reasons at present undefined, may be one of the more sensitive species to inorganic arsenic-induced cancers (Goering et al., 1999). Inorganic arsenic increases the incidence of skin papillomas in Tg.AC mice (that carry the *v-Ha-ras* oncogene), but only after treatment with a tumor promoter and not when given alone (Germolec et al., 1998). Inorganic arsenicals can undergo extensive metabolism and undergo reduction and/or methylation in many tissues (Goering et al., 1999). The organo-arsenical, dimethylarsinic acid (DMA), can be a tumor promoter in rats in two-stage carcinogenesis studies using organic carcinogens as the initiators (Yamamoto et al., 1997) or a complete carcinogen in the male rat bladder (Wei et al., 1999). DMA is much less acutely toxic than inorganic arsenicals and the high level of DMA required for carcinogenic effect in most of these studies (Yamamoto et al., 1997) raises the question of relevance with regard to the actual attainable doses of inorganic arsenic (NRC, 1999). In any event, the carcinogenicity in animals of inorganic arsenic when given as a single agent is considered equivocal or limited (IARC, 1980, 1987; NRC, 1999). For example, one study by Osswald and Goerttler (1971) gave repeated intravenous (i.v.) injections of sodium arsenate to female Swiss mice over 24 months. Of 19 arsenate-treated mice having undergone postmortem examination, 11 had hematopoietic tumors, compared to none in 16 examined untreated controls (Osswald and Goerttler, 1971; IARC, 1980). Unfortunately, this study was reported prior to its completion and a substantial number of the control animals were still alive at the time of reporting (Osswald and Goerttler, 1971; IARC, 1980). No subsequent reporting has occurred. The early reporting while control animals were still alive was considered to make these results difficult to interpret and was appraised as a noteworthy inadequacy in study design (IARC, 1980). In addition, there was no vehicle treated control group.

Thus, the goal of this work was to determine if repeated i.v. injections of sodium arsenate in Swiss mice are carcinogenic when compared to appropriate control groups under the more rigorous conditions of modern bioassays and at a point at which all animals had been terminated and examined. The results of this study have been published in the primary literature (Waalkes et al., 2000) and are summarized again here.

MATERIALS AND METHODS

Animals and Treatment

Groups of 50 male and 50 female 4-weeks-old Swiss [CR:NIH(S)] mice were obtained from the Animal Production Area, NCI-Frederick, Frederick, MD, and housed five per hanging cage. They were provided food and water ad libitum.

For injection, sodium arsenate (dibasic; Na_2HAsO_4; J.T. Baker Co.) solutions were

prepared in sterile normal saline. At 8 weeks of age animals were divided into gender-matched groups of 25 and treated intravenously (i.v.; 10.0 ml/kg) with 0.5 mg arsenate/kg once weekly in the tail vein for 20 consecutive weeks. The schedule of injections and dosage duplicated the study of Osswald and Goerttler (1971). Controls received saline i.v. at 10 ml/kg. The week of the first injection was designated as experimental week 1. Body weights, survival, and clinical signs were recorded throughout the experiment. Clinical signs were checked daily. Animals were killed when significant clinical signs developed or at 96 experimental weeks.

Pathology

An extensive necropsy was performed on all animals whether found dead, sacrificed during the experiment when appropriate clinical signs developed, or sacrificed at the conclusion of the experiment. Kidney, liver, lung, spleen, lymph nodes (mandibular, mesenteric), testis, coagulating gland, urinary bladder, seminal vesicles, uterus, ovaries and all abnormal tissues from each animal were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histological examination. Skin was carefully inspected on all parts of the animal and any abnormal lesions were taken and processed as above. Upon the discovery of excess uterine proliferative lesions in the arsenate-treated mice, lesions were re-evaluated using the criteria described earlier (Nagaoka et al., 1994; Diwan et al., 1997). All pathological assessments were performed in a blind fashion.

Immunohistochemical Analysis

Proliferating cell nuclear antigen (PCNA) was used as an indication of cellular proliferation using the method described in Waalkes et al. (2000). The specimens included nine uterine hyperplastic lesions (three mild and six moderate to severe cases) from treated animals and five cases (two normal, two mild and one moderate to severe) from control mice and one adenocarcinoma (from an arsenate exposed animal). Sections from these same cases were also used to evaluate the immunohistochemical localization and intensity of estrogen receptor (ER-α), and cyclin D1 using the methods described in Waalkes et al. (2000). The staining intensity of the cells was categorized as negative (−), weak (+), moderate (++) or strong (+++), while the extent of staining was scored as 1 if fewer than 25% of cells were positive, 2 if 25 to 75% of cells were positive, and 3 if more than 75% of the cells were positive.

Data Analysis

In all cases a one-sided probability level of $P \leq 0.05$ was considered to indicate a significant difference. In pairwise comparison of lesion incidence or survival at the study termination, Fisher's exact test was used. For severity ratings and body weight data, a Student's *t*-test was used to compare means. Survival during the study was examined with the Cox test and the generalized Kaplan–Meier test and was considered significantly different only if so indicated by both tests.

RESULTS

Repeated injections of arsenate in male and female Swiss mice did not alter the number of animals surviving at the termination of the study. In addition, both the Kaplan–Meier and the Cox methods for defining differences in survival also indicated no significant differences in survival between treated mice and gender-matched controls. Furthermore, body weights were unaltered by arsenate treatment during the study.

Table 1 shows the effects of arsenate injections on lesions in the uterus of female mice. Arsenate sharply increased the incidence (2.8-fold) and severity (moderate/severe

TABLE 1

Effect of repeated injections of sodium arsenate on the incidence and severity of cystic hyperplasia of the uterus in female Swiss mice

Group	Hyperplasia	
	Incidence (%)	Severity
Control	5 (20%)	0.56 ± 0.24
Arsenate	14 (56%) [*,a]	1.64 ± 0.32 [*]

Note: Both groups consisted of 25 mice and were treated with one i.v. injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals) for incidence and mean ± SEM score for severity based on the following severity ratings: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe. An asterisk (*) indicates a significant difference from control by Fisher exact test (incidence) or Student's *t*-test (severity).

[a] The arsenate-treated group also had a malignant adenocarcinoma of the uterus. Total proliferative lesion (hyperplasia + adenocarcinoma) incidence would then equal 60%.

lesions; 2.9-fold) of cystic hyperplasia of the uterus compared to control. Uterine cystic hyperplasia is considered to be a precursor to adenocarcinoma (Johnson, 1987; Newbold, 1995; Diwan et al., 1997) and, indeed, in the arsenate-treated group a relatively rare uterine adenocarcinoma occurred. The greatly elevated incidence of preneoplastic lesions of the uterus points towards arsenate as a causative factor in the adenocarcinoma, although this cannot be definitively established by the present data.

Because of this marked increase in the incidence of preneoplastic lesions of the uterus with arsenate and the occurrence of an adenocarcinoma of the uterus in an arsenate-treated animal, additional study was directed at a more complete characterization of these uterine lesions. In this regard, PCNA was used to gauge the level of cellular proliferation within uterine lesions and adjacent normal tissue. In normal uterine tissue, the intensity of PCNA staining was weak and less than 25% of the cells showed positive staining (Table 2). In hyperplastic epithelium of uterus in the control mice, PCNA staining generally showed less intensity than in arsenate-treated uterine hyperplasias. The levels of cyclin D1 were also measured in uterine tissue, since these cyclins are essential for the progression through the G1 phase of the cell cycle, they too are considered indicators of cell proliferation. Immunohistochemical analysis in control uterine tissue showed minimal staining for cyclin D1 (Table 2). Levels of nuclear staining for cyclin D1 were, however, higher in the hyperplastic and neoplastic epithelium of arsenate-treated mice than in those of control mice. These findings suggest that cyclin D1 overexpression precedes or is concurrent with cellular transformation.

ER-α was detected in both epithelial and stromal cell nuclei of uterine tissue. The ER is a hormone-activated transcription factor that mediates the biological effects of estrogens in a variety of target tissues and is often overexpressed in tumors of endocrine sensitive tissue (Barton and Shapiro, 1988). The expression of ER-α was weak to moderate in normal uteri. Early hyperplastic lesions induced by arsenate showed moderate to strong expression of ER-α, in comparison to control lesions as the number of ER-α-positive epithelial cells and staining intensity increased in the hyperplastic epithelium of arsenate-treated rats (Waalkes et al., 2000). In female mice there was a definite increase in the incidence of total proliferative hepatocellular lesions (adenomas and foci of cellular alteration) in arsenate-treated female mice compared to gender-matched controls (Table 3). The incidence of hepatic proliferative lesions was unaffected by arsenate exposure in male mice.

Repeated arsenate injections in male mice doubled the incidence of interstitial cell hyperplasia and significantly increased their average severity rating (Table 4). This was

TABLE 2

Immunoreactivity for PCNA and cyclin D1 in uterine epithelium of Swiss mice exposed to sodium arsenate

Treatment	Uterine hyperplasia	PCNA		Cyclin D1	
		Intensity	Extent	Intensity	Extent
Arsenate	mild	+++	2	+++	3
Arsenate	mild	++	2	++	3
Arsenate	mild	+++	2	+++	2
Arsenate	moderate to severe	++	2	++	3
Arsenate	moderate to severe	+++	3	+++	3
Arsenate	moderate to severe	++	1	++	3
Arsenate	moderate to severe	++	2	+++	2
Arsenate	moderate to severe	++	1	++	3
Arsenate	moderate to severe	++	2	+++	2
Arsenate	adenocarcinoma	++	2	+++	2
Control	absent	–	–	+	1
Control	absent	–	–	+	1
Control	mild	++	1	+	1
Control	mild	+	2	+	2
Control	moderate to severe	++	2	++	2

Note: Intensity: – = negative; + = weak; ++ = moderate; +++ = strong. Extent: 1 = 1–25% of cells per lesion stained; 2 = 25–75% of cells per lesion stained; 3 = 75–100% of cells per lesion stained.

TABLE 3

Effect of repeated injections of sodium arsenate on the incidence of proliferative hepatocellular lesions of the liver in male and female Swiss mice

Group	Foci of alteration	Adenoma	Carcinoma	Total tumors	Total proliferative lesions
Male control	1 (4%)	0	0	0	1 (4%)
Male arsenate	1 (4%)	0	0	0	1 (4%)
Female control	1 (4%)	0	0	0	1 (4%)
Female arsenate	5 (20%)	1 (4%)	0	1 (4%)	6 (24%)*

Note: All groups consisted of 25 mice and were treated with one i.v. injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals). An asterisk (*) indicates a significant difference from control by Fisher exact test.

TABLE 4

Effect of repeated injections of sodium arsenate on the incidence and severity of testicular interstitial cell hyperplasia and tubular degeneration in male Swiss mice

Group	Hyperplasia		Tubular degeneration	
	Incidence (%)	Severity	Incidence (%)	Severity
Control	8 (32%)	0.72 ± 0.20	20 (80%)	2.48 ± 0.28
Arsenate	16 (64%)*	1.28 ± 0.18*	17 (68%)	2.20 ± 0.34

Note: Both groups consisted of 25 mice and were treated with one i.v. injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals) for incidence and mean ± SEM score for severity based on the following severity ratings: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe. An asterisk (*) indicates a significant difference from control by Fisher exact test (incidence) or Student's *t*-test (severity).

TABLE 5

Effect of repeated injections of sodium arsenate on the incidence of skin hyperkeratosis in male and female Swiss mice

Group	Incidence of hyperkeratotic skin lesions (%)
Control males	1 (4%)
Arsenate males	5 (20%) [a]
Control females	0 (0%)
Arsenate females	9 (38%) *

Note: All groups consisted of 25 mice and were treated with one i.v. injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals). An asterisk (*) indicates a significant difference from control by Fisher exact test.

[a] The incidence of hyperkeratotic skin lesions in the arsenate-treated males approached significance when compared to gender-matched controls by Fisher exact test ($P = 0.095$).

not associated with an increase in the incidence or severity of testicular tubular degeneration, a lesion which can be associated with indirectly induced testicular interstitial cell hyperplasia.

Hyperkeratotic skin lesions were also produced by the repeated injection of arsenate (Table 5). In males the incidence of these skin lesions was increased from 4% in controls to 20% with arsenate (not significant) whereas in females the incidence increased from 0% in controls to 38% with arsenate exposure (highly significant). One of the primary target sites in human arsenic carcinogenesis is the skin.

Pulmonary tumors are also associated with arsenic exposure in humans. However, repeated injections of arsenic had no effect on lung tumors, although these tumors were quite common (Waalkes et al., 2000). For the hematopoietic system there were 5 lymphomas in the 25 control female mice and 3 lymphomas in the 25 arsenate-treated female mice. In male mice there was one lymphoma in controls and one in arsenate-treated animals. Other sites proved unremarkable with regard to proliferative lesions.

DISCUSSION

The results of this study indicate that repeated arsenate injections are associated with proliferative, pre-neoplastic lesions of the reproductive tissues in Swiss mice, specifically the uterus and testis, and of the female liver (Waalkes et al., 2000). Additionally, an endometrial adenocarcinoma occurred after arsenate exposure. This event should be placed in the context of the rarity of spontaneous uterine adenocarcinomas in mice of Swiss background (~0.5%; Percy and Jonas, 1971). Although not allowing a definitive conclusion to be drawn, a causal role for arsenate is implicated. Our study failed to confirm previous results of a link between repeated arsenate injections and tumors of the hematopoietic system (Osswald and Goerttler, 1971). The reasons for this are not immediately clear. Arsenate exposure was also associated with hyperkeratototic skin lesions in female mice. The skin is a well recognized target site of arsenic toxicity in humans (IARC, 1980; NRC, 1999) and rodents (Germolec et al., 1998).

The strong association seen between incidence and severity of uterine hyperplasia and arsenate exposure, and its possible association with adenocarcinoma, are intriguing. Uterine hyperplasia is an early stage in a continuum of changes leading eventually to endometrial adenocarcinoma (Crum, 1994). Uterine hyperplasia is often related to an abnormally high, prolonged estrogenic stimulation, frequently in the absence of progestational activity (Crum, 1994). Thus, uterine hyperplasia is associated with diminished

ovarian function and anovulatory syndromes (Crum, 1994). Because these uterine lesions were unexpected in this study, assessment of ovarian function, such as through menstrual cycling, was not performed. No overt lesions occurred in the ovaries of arsenate-treated mice indicative of dysfunction, but this does not eliminate the possibility of diminished function. One study, in fact, indicates that arsenic treatment in freshwater fish can impair ovarian function (Shukla and Pandey, 1984), but how this might apply to mammals is unclear. On the other hand, exogenous estrogen can supply the impetus for proliferative neoplastic progression in the uterus and many studies indicate that steroid hormones and xenoestrogens can produce preneoplastic and neoplastic lesions in estrogen responsive tissues, including uterus, in humans and experimental animals (Johnson, 1987; Crum, 1994; Davis and Bradlow, 1995; Newbold, 1995; Diwan et al., 1997; Korach et al., 1997; Zava et al., 1997; Persson et al., 1999; Weiderpass et al., 1999). Under this hypothesis arsenate would somehow be acting directly or indirectly to stimulate estrogen activity in the uterus. In this regard, the ER-α, a member of the estrogen receptor superfamily, is a hormone-activated transcription factor that mediates the biological effects of estrogens in a variety of target tissues (Musgrove and Sutherland, 1994; Beato et al., 1995). In responsive tissues, the ER stimulates the expression of specific estrogen-regulated genes (Ignar-Trowbridge et al., 1992; Musgrove and Sutherland, 1994; Newbold, 1995; Korach et al., 1997). The sensitivity of a given tissue to estrogens varies with the levels of estrogen receptor (Korach et al., 1997). In the present study early hyperplastic lesions induced by arsenate showed moderate to strong expression of ER-α, in comparison to control lesions. All these uterine lesions, as well as more severe hyperplastic lesions of arsenate-treated mice, were also strongly positive for cyclin D1 and PCNA. Both PCNA and cyclin D1 are indicators of enhanced cell proliferation. Beyond this, several studies suggest that cyclin D1 may be involved in mediating steroid-dependent growth of both normal and malignant epithelial cells (Buckley et al., 1993; Sicinski et al., 1995). Moreover, a strong expression of cyclin D1 along with ER overexpression has also been seen in malignant breast cells (Musgrove and Sutherland, 1994). Thus, estrogen may play a direct role in regulation of cyclin D1 expression. In any event, it appears that arsenate may be associated with estrogen-related genetic events in the uterus and that these events may lead to hyperplasia.

The hyperplastic interstitial cell lesions of the testes seen with arsenic treatment in the present work could also be consistent with an estrogen-related mechanism. It is quite clear that chronic estrogenization can induce proliferative interstitial cell lesions of rodent testes (Huseby, 1976; Bosland, 1994, 1996; Thomas, 1997). Other inorganic compounds can induce interstitial cell proliferations in rodents but do so in the presence of severe testicular degeneration (Bosland, 1994) and with loss of androgen production and apparent subsequent hypergonadotrophic stimulation of remnant testicular tissue (Waalkes et al., 1997). This is not consistent with the observed absence of degenerative testicular lesions in the present study with arsenic. Several studies indicate that factors localized within the testes are critical to testicular proliferative lesion formation induced by estrogens (Huseby, 1976; Bosland, 1994). Under these circumstances arsenic could be either enhancing estrogen production or otherwise enhancing its actions, reducing estrogen degradation or otherwise prolonging its actions, or acting as an estrogen itself. Arsenic has been shown to interact with certain steroid receptors, although this reduces activity (Simons et al., 1990), so action as a direct steroid mimic seems unlikely. Otherwise little is known about how arsenic might enhance or prolong the effects of estrogenic compounds. The activity of arsenic methyltransferase, the enzyme which methylates arsenic, is quite high in the mouse testis (Healy et al., 1998), but how this might relate to hyperplasia development is unclear. Additional study will be required to define the possible role of arsenic in any estrogenic stimulation of testes and uterus.

The arsenate-treated female mice in our study showed an increased incidence of hepatic proliferative lesions, including preneoplastic hepatocellular foci of alteration and a

hepatocellular adenoma. Arsenic exposure can induce liver hyperplasia in rainbow trout (Kotsanis and Iliopoulou-Georgudaki, 1999) and there is evidence linking arsenic and liver cancer in humans (NRC, 1999). The occurrence of such liver lesions in the present study is noteworthy because Swiss mice are refractory to spontaneous or chemical-induced liver tumor development (Diwan and Meier, 1976). Exactly how arsenate induces hepatocellular lesions is not known, but it could be related to some sort of estrogenic action as estrogenic hormones are well-established hepatocellular carcinogens and liver tumor promoters (Schuppler and Günzel, 1979; Moolgavkar, 1986; Metzler and Degen, 1987). Thus, the finding of proliferative liver lesions in the present study may also be indirect evidence of a potential estrogenic mechanism of action for arsenate, although such liver lesions can be induced by many other mechanisms.

In summary, the results of this study clearly show that repeated exposures to arsenate can induce hyperplastic lesions in the mouse uterus. This occurs in the context of increased ER-α expression and estrogen receptor-associated gene products, like cyclin D1, which are overexpressed in arsenate-induced proliferative uterine lesions. These results suggest, but do not establish, that the high incidence of uterine proliferative lesions observed in the present study may somehow be caused by arsenic-mediated modulation of estrogen functions. The finding of interstitial cell hyperplasia in a large percentage of arsenate-treated animals tends to support this argument, since estrogens are known testicular tumorigens (Newbold et al., 1986, 1987). Induction of proliferative lesions of the female liver is also consistent with estrogen exposure (Schuppler and Günzel, 1979; Moolgavkar, 1986), although many mechanisms can lead to liver hyperplasia. The precise fashion in which arsenic might alter the endogenous level and/or half-life of estrogenic steroid hormones is undefined. Issues of route of exposure and dosage frequently complicate extrapolation from rodent studies to humans, and the present study is no exception. However, the possible action of arsenic as a xenoestrogen deserves additional study.

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The results discussed within this report have been previously published in the primary literature (Waalkes et al., 2000) and are summarized again here with the approval of the publisher, Academic Press.

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Arsenic Exposure and Health Effects IV
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Sub-toxic Arsenite Induces a Multi-component Protective Response against Oxidative Stress in Human Cells

Elizabeth T. Snow, Michael Schuliga, Salem Chouchane, Yu Hu

ABSTRACT

We have investigated the effect of arsenite, As(III), on cultured human keratinocytes and fibroblasts looking at the activity of glutathione-related enzymes, DNA repair function, and the expression of both redox-related and DNA repair genes. We have found that genes related to both cellular redox control and base excision repair are coordinately up-regulated by very low (essentially non-toxic) doses of As(III). The DNA repair genes are then down-regulated by slightly higher doses of As(III). This correlates with our finding that pre-treatment with arsenite can modulate the toxicity of DNA-damaging agents in a manner that is consistent with the induction of a protective response by a very low dose of As(III) combined with synergistic toxicity at higher doses. Our data provide strong evidence that a low dose of As(III) provokes a multi-faceted protective response against further insults of oxidative stress. Yet, at slightly higher doses arsenite exhibits strong synergistic toxicity with these same agents. These results suggest that caution is warranted when assessing risk due to arsenic exposure among human populations since we do not know if long-term exposure might not lead to persistent changes in cellular regulation or homeostasis. It is clear, however, that a linear extrapolation of risk based on exposure to high, toxic concentrations of arsenic cannot adequately predict response to low, sub-toxic concentrations.

Keywords: arsenate; enzyme inhibition; keratinocytes; cell culture; DNA repair; redox; oxidative stress

INTRODUCTION

Chronic 'low dose' exposure to arsenic in the environment causes a multitude of adverse health effects in humans, ranging from altered skin pigmentation to peripheral vascular disease and cancer (Shannon and Strayer, 1989; Wu et al., 1989; Byrd et al., 1996; Chen et al., 1996). Although single exposures to arsenic may be sufficient to trigger some of these adverse health effects, in general most arsenic-related disease is the result of chronic exposure to sub-lethal concentrations of inorganic arsenic, in either its trivalent, As(III), or pentavalent, As(V), form. As(V) is effectively reduced to As(III) in vivo. As(III) is the most toxic form of inorganic As and is considered to be the form which is most likely to provoke carcinogenicity. Doses of arsenic sufficient to kill large numbers of cells provoke acute toxicity and are not generally encountered in the environment. Both cancer and vascular disease start in proliferating cells, although vascular disease could also result from toxicity to some cells that results in a proliferative response in other cells (such as atherosclerotic plaque formation). Most of the chronic heath effects of arsenic occur due to changes in the epithelial or endothelial cells that predominate in target tissues, such as skin, bladder, lung, and the peripheral vascular system. We therefore evaluated the effects of As(III) in human epithelial keratinocytes and fibroblasts at concentrations that could be encountered by these cell types during chronic exposure to environmental arsenic.

Cellular Redox Control

Arsenic in its trivalent and most toxic forms acts predominantly by virtue of its interactions with cellular thiols. Oxidation of thiols and cross-linking of closely positioned dithiols will alter the redox status of the affected proteins and peptides. Cell signalling processes known to be affected by As(III) are likely to be mediated by the oxidation or reduction of appropriate thiol groups. Whereas some endogenous thiols provide an effective antioxidant defense against these changes, arsenite may selectively promote the oxidation or cross-linking of susceptible thiols. Cellular thiols critical for the overall cellular redox status include the tripeptide glutathione (GSH) and the sulfur-rich protein, thioredoxin (Trx). Other enzymes involved in the synthesis and maintenance of these redox proteins include glutathione reductase (GR), glutathione peroxidase (GPx), and thioredoxin reductase (TR). We show here that 24 h exposure of human fibroblast and keratinocyte cells to low dose arsenite causes the coordinate up-regulation each of these proteins, except GPx, which is down-regulated.

Arsenic and DNA Repair

Another aspect of arsenic toxicity is the production of oxidative stress and an acute burst of reactive oxygen species. Even low doses of As(III) provoke this oxidative burst (Liu and Jan, 2000). Moderate doses of arsenic are also known to inhibit DNA repair (Li and Rossman, 1989; Hartwig et al., 1997; Lynn et al., 1997; Hu et al., 1998). Base excision DNA repair (BER) is a short-patch DNA repair process that is critical for the repair of oxidative DNA damage. Enzymes required for BER include damage-specific DNA glycosylases, apurinic/apyrimidinic (AP) endonuclease, DNA polymerase β, and DNA ligase. DNA polymerase β (Chen et al., 1998) and AP endonuclease (Harrison et al., 1996) have both been identified as rate-limiting enzymes for this process. We have previously shown that DNA repair enzymes are not direct targets for As(III)-dependent enzyme inhibition. We report here that a 24 h exposure of human fibroblasts to sub-micromolar concentrations of As(III) causes a significant up-regulation of the genes for AP endonuclease (h*APE*1) and DNA polymerase β (*polb*) while higher doses (>5 μM) induce a dose-dependent down-regulation. When 24 h pre-treatment with As(III) is followed by exposure to a second agent which produces DNA damage that can be repaired by BER, the As(III) pre-treatment provokes a protective response at low dose and synergistic toxicity at a higher dose.

These results are summarized, placed into the context of human exposure, and a model for the molecular response of human cells to low dose arsenic is proposed.

METHODOLOGY

Chemicals

Sodium arsenite, reduced glutathione (GSH), oxidized glutathione (GSSG), ATP, *cis*-dinitrobenzene (CDNB), neutral red dye, *N*-acetylcysteine (NAC), L-cystine, purified glutathione S-transferase (GST), and GSH peroxidase (GPx) were purchased from Sigma (St. Louis, MO). NADPH and GSSG reductase (GR) were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Polyclonal antiserum against human DNA polymerase β was a gift from Dr. Samuel Wilson, NIEHS. Monomethylarsonous acid, MMA(III), was synthesized using the method of Burrows and Turner (1920) and Goddard (1930) (Chouchane and Snow, 2001).

Cell Culture

SV40 transformed human keratinocytes (AG06 cells) were obtained from Dr. Mark Steinberg at City College of New York. WI38 human fibroblasts were obtained from either the ATCC or from CSL (Victoria, Australia) and GM847 immortalized human fibroblasts were obtained from the Murdoch Institute (Melbourne, Australia). Keratinocyte and fibroblast cells were grown in Dulbecco's Modified Eagle medium (DMEM) and Basal Modified Eagle medium (BME), respectively, each containing 10% fetal bovine serum, 2 mM glutamate, 100 Units penicillin-G, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Some fibroblast cultures were grown in sealed flasks at 37°C without additional CO_2. Cell viability was determined by neutral red dye uptake, as described previously (Snow et al., 1999).

Enzyme Assays

For assays of enzyme activity related to GSH metabolism, 2×10^5 cells were seeded into a six-well plate and allowed to grow for 48 h. The cells were then exposed to As(III) for the time indicated. Treated cells were collected by trypsin digestion, washed twice with cold phosphate buffered saline, and isolated by centrifugation. The cells were homogenized by microtip sonication and the supernatant was used to determine protein concentration and enzyme activity. Protein was assayed by the Bio-Rad technique using bovine serum albumin as the standard.

Glutathione Reductase (GR)

GR activity was determined by monitoring the disappearance of NADPH at 340 nm in the presence of GSSG. Cell extracts were preincubated in 0.15 M phosphate buffer (pH 7) containing 6 mM EDTA and 0.1 mM GSSG at 37°C for 2 min. The reaction was started by the addition of 0.23 mM NADPH. The net rate of enzymatic oxidation of NADPH was used to calculate enzyme activity (Styblo and Thomas, 1995).

Northern Analysis

Total cellular RNA was isolated using the QIAGEN RNeasy kit according to the manufacturer's instructions. RNA fractionation on agarose–formaldehyde gels and blot transfer using nitrocellulose membranes was performed as described by Sambrook et al. (1989). Probes made by polymerase chain reaction amplification of cDNA were labelled with radioactive $\alpha[^{32}P]$-dCTP using random oligonucleotides as primers and used to detect the mRNA. Hybridized blots were exposed to X-ray film at −70°C. The developed films were scanned using a Bio-Rad G710 densitometer, and analyzed using Bio-Rad Quantity One software.

Western Blots

Whole-cell protein extracts from treated cells were prepared as described above. Nuclear and cytoplasmic protein extracts were also prepared from WI38 cells by differential centrifugation. Proteins (20 μg per lane) were separated by electrophoresis on denaturing SDS-polyacrylamide gel electrophoresis and the proteins were transferred to Hybond-ECL membrane by semi-dry blotting. The amount of protein was determined by binding of specific antisera prepared in rabbits and visualized by binding of horseradish peroxidase-conjugated secondary anti-rabbit antibody using the Boehringer Mannheim chemiluminescent blotting substrate.

Statistical Analysis

All assays were repeated at least once and the results are expressed as the mean (± the standard error of the mean, where appropriate). Student's *t*-test was used to determine statistical significance of the measurements relative to controls.

RESULTS

In order to determine the dose range appropriate for measurements of effect in viable cells, several human cell lines were assayed for arsenic toxicity after acute or sub-chronic As(III) exposures of 3 to 72 h. All fibroblast and keratinocyte cell lines examined showed 50% toxicity as measured by neutral red dye uptake after a 24 or 48 h exposure to concentrations of As(III) in the range of 5 to 50 μM (Table 1). We also tested the toxicity of MMA(III) in SV40-transformed human keratinocytes (AG06 cells). After a 48 h exposure, MMA(III) is equitoxic to human keratinocytes compared to inorganic As(III) (Fig. 1). However, the rate of killing by MMA(III) is higher than that of As(III). Less than 20 μM of MMA(III) is required to kill 50% of the cells after a 5 h exposure whereas a similar 5 h exposure to As(III) only produces 20% toxicity. This suggests that MMA(III) is taken up by the cells more rapidly than As(III), but is not indicative of a different mechanism of action for the two forms of arsenic. It is also consistent with other data that suggest that toxicity is related to inhibition of mitochondrial function (Petrick et al., 2000) and that MMA(III) reaches the mitochondrial compartment more rapidly than As(III).

Arsenic is known to induce a cellular stress response with a resultant increase in heme oxygenase and other heat shock proteins (Parrish et al., 1999). Glutathione levels are also increased in some cells (Ochi, 1997; Snow et al., 1999). We have previously found that physiologically relevant concentrations of arsenic, in a variety of forms, do not directly

TABLE 1

Toxicity of As(III) to human keratinocytes and fibroblasts

Cell line	IC_{50} (μM)	
	24 h	48 h
AG06	22.9 ± 5.3	7.2 ± 1.0
HaCaT	20.6 ± 0.5	11.6 ± 0.9
NHEK	27.5 ± 4.1	12.3 ± 1.2
GM847	26.7 ± 4.0	10.7 ± 5.5
WI38	51.3 ± 9.4	11.2 ± 1.9

Human keratinocyte (AG06, HaCaT, and NHEK) or fibroblast (GM847 and WI38) cells were exposed to As for 24 or 48 h and cell viability was determined by neutral red dye uptake, as described in the Methods section. The concentration of As(III) in μM required to reduce dye uptake to 50% of the unexposed control (IC_{50}) was determined by least-squares fit of a semilog plot of the data. All values are the average of 2 or more separate experiments with 3 or more replicates per experiment.

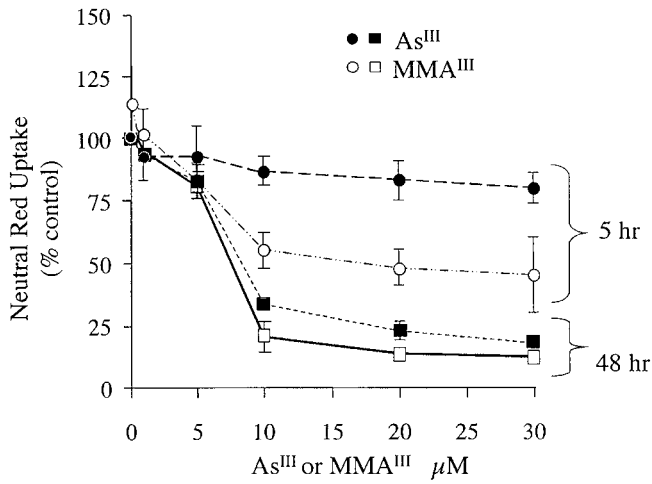

Fig. 1. Relative toxicity (measured by neutral red dye uptake) of monomethyl arsenite, MMA(III), and inorganic arsenite, As(III), in AG06 cells after a 5 h or a 48 h exposure.

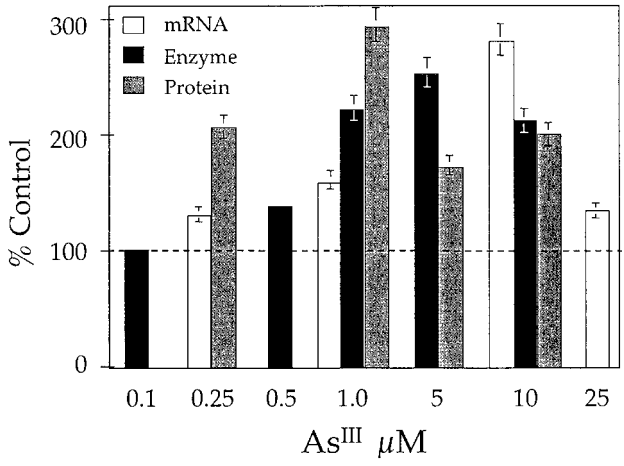

Fig. 2. Glutathione reductase mRNA levels, enzyme activity, and protein levels in GM847 or AG06 cells after 24 h treatment with As(III). The mRNA concentration in GM847 cells was determined by Northern blot assay using a 297 base pair cDNA probe prepared by RT-PCR. The enzyme activity of glutathione reductase was determined using total cell extracts by the method of Styblo and Thomas (1995), as described in the Methods section. Protein levels were measured in AG06 cells by Western blot using a polyclonal antibody obtained from R.H. Schirmer, University of Heidelberg. All measurements were converted to percent of control and the results shown represent the average of four separate experiments ± the standard error of the mean (sem).

inhibit GSH metabolizing enzymes (Chouchane and Snow, 2001). To see if the expression of these and other GSH-related genes is altered in skin cells, we measured the activity of GSH-related enzymes in As(III)-exposed human epidermal keratinocytes and fibroblasts. We have found that several enzymes, including GR, are significantly up-regulated by exposure of the cultured cells to up to 10 μM As(III) for 24 h (Fig. 2). This up-regulation results in a 3-fold increase in mRNA and protein levels, and a 2.5-fold increase in GR enzyme activity. Other genes, notably Trx and TR, are up-regulated in parallel with GR (Fig. 3). However, glutathione peroxidase (GPx) is down-regulated by similar arsenic exposures. GPx enzyme activity is also decreased in AG06 cells after 24 h exposure to 0.2 to 2 μM As(III) (not shown). GST activity is increased in both fibroblasts and keratinocytes after exposure to higher doses of As, but not as significantly or as consistently as the other genes (not shown).

Base excision DNA repair enzymes and BER genes are also up-regulated by low sub-

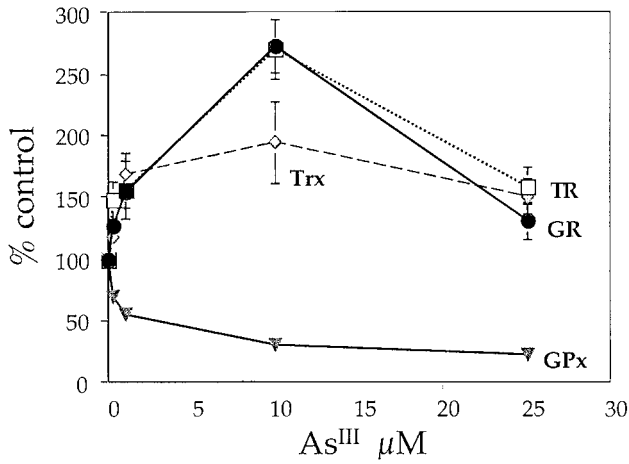

Fig. 3. Increased expression of thioredoxin (Trx), thioredoxin reductase (TR), glutathione reductase (GR) mRNA, and the parallel decreased expression of glutathione peroxidase (GPx) mRNA after 24 h exposure of GM847 cells to increasing As(III). Messenger RNA levels were assayed by Northern blot analysis, and the average optical density values were converted to percent of untreated control. The results show the average ± sem for four separate experiments.

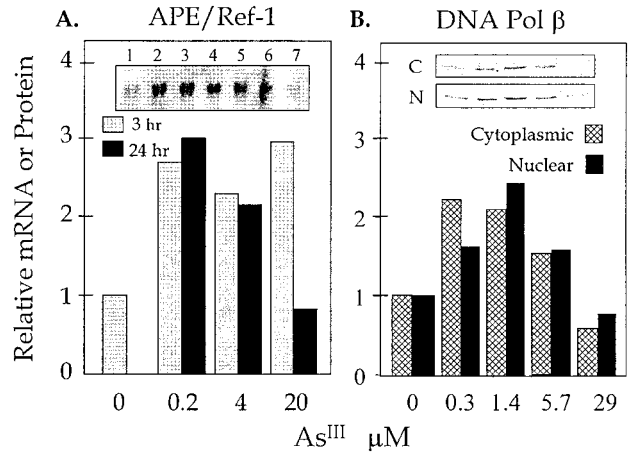

Fig. 4. Up-regulation of BER genes in human keratinocytes and fibroblasts. (A) APE/Ref-1 mRNA in AG06 keratinocytes is shown on the left. The Northern blot (inset) indicates the relative amount of APE mRNA present in (1) untreated control cells (2,4,6) after 3 h exposure to 0.2, 4, or 20 μM As(III), and (3,5,7) after 24 h exposure to 0.2, 4, or 20 μM As(III). (B) DNA polymerase β protein levels in cytoplasmic and nuclear extracts from As(III)-exposed WI38 cells as determined by Western blotting are shown on the right. Single representative experiments are shown.

toxic concentrations of As(III) (Fig. 4). At least three BER enzymes are up-regulated in parallel by 0.1 to 20 μM As(III), DNA ligase I (not shown), AP endonuclease (APE/Ref-1), and DNA polymerase β. Up-regulation of APE/Ref-1 in AG06 cells has been measured at the level of RNA transcription (Fig. 4A) and protein concentration (not shown). A very similar increase in APE/Ref-1 mRNA was obtained in normal human epidermal keratinocytes (NHEK cells) in which the expression peaked after 6 h of As(III) at a concentration of 4 μM (not shown). An increase in DNA polymerase β protein concentration was seen both in nuclear extracts and in cytoplasmic extracts from WI38 cells exposed to 0.3 to 1.4 μM As(III) (Fig. 4B). DNA ligase I exhibited a similar 2-fold increase in protein in nuclear extracts from WI38 cells and the total ligase activity per μg protein was increased a similar amount (not shown). Surprisingly, the optimum increase in expression of the DNA repair genes occurs at 10- to 20-fold lower concentrations of As(III) than the redox-related genes shown above.

We have previously shown that pre-treatment of AG06 cells with sub-micromolar concentrations of As(III) protects against subsequent exposure to a methylating agent,

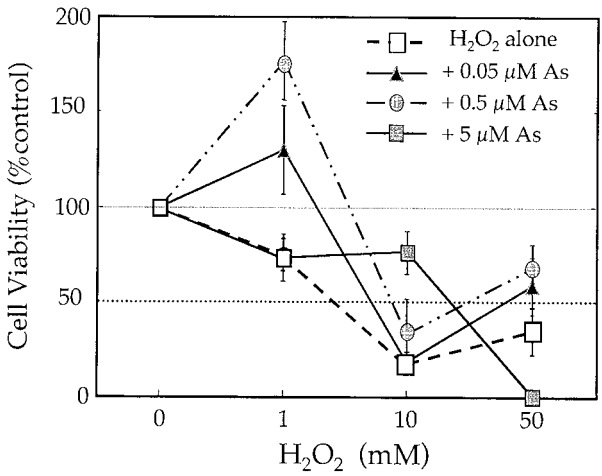

Fig. 5. Pretreatment with a low dose of As(III) for 24 h protects cells against killing by H_2O_2. WI38 cells were plated in 96-well plates then incubated with medium alone or medium plus 0.05, 0.5, or 5.0 μM As(III) for 24 h. The cells were then exposed to 0, 1, 10, or 50 mM H_2O_2 for 60 min in the absence of serum. The cells were washed, given fresh complete media, and allowed to recover for an additional 24 h. Cell viability was assayed by neutral red dye uptake. All values represent the average of 4 measurements ± sem.

MNNG (Snow et al., 1999). Alkylation damage is typically repaired by the BER pathway. We now ask the question, does exposure to low dose As(III), an agent that induces oxidative stress, protect against oxidative DNA damage repaired by the BER pathway? To test this we exposed WI38 fibroblasts to As(III) for 24 h and then exposed the cells to H_2O_2 to produce oxidative DNA damage. In two separate experiments we found that exposure to sub-micromolar, but not 5 μM As(III) significantly ($P < 0.05$) protected the cells against toxicity induced by 1 mM H_2O_2 (Fig. 5).

DISCUSSION

Dose–response is of critical importance for assessment of arsenic toxicity. Arsenic is not a direct acting carcinogen. It acts as a tumor promoter or progressor, presumably by invoking a persistent cellular stress response. Both the metabolism and the molecular response to arsenic vary with dose and with cell type. A very low dose of arsenic may induce endothelial cell proliferation (Germolec et al., 1997), whereas a slightly higher dose may trigger changes in the regulation of BER, as shown here. Up-regulation of BER correlates with increased cell viability in the face of a challenge dose of an alkylating or oxidizing DNA damaging agent. However, exposure to slightly higher doses of As(III) (above one micromolar) causes down-regulation of the BER genes as well as parallel up-regulation of a variety of redox-related enzymes. Thus even low, relatively non-toxic concentrations of As(III) can directly modulate cellular redox levels which, in turn, may alter cellular signalling and other aspects of intermediary metabolism and thereby contribute to the carcinogenic process. Sub-acute exposure (24 to 48 h) to low micromolar concentrations of As(III) coordinately up-regulates GSH, Trx, and other SH-dependent redox enzymes, such as TR and GR. This response to a moderate dose of arsenic is expected to provide additional protection against oxidative stress by improving the redox balance of the cell, but at the expense of decreased BER capacity. This may cause increased mutagenesis in a proliferating population of cells (Li and Rossman, 1991). Thus there is apparently a very narrow window for the induction of a true protective response.

These changes in gene expression and subsequent protective effects occur at doses of arsenic that are consistent with a range of exposure that may be reached in vivo. As shown in Fig. 6, the levels of arsenic found in the drinking water of highly exposed human populations, such as in Bangladesh, range from 0.2 to 25 μM. The changes that we see in DNA repair enzymes occur after exposure of cultured cells to 0.1 to 5 μM

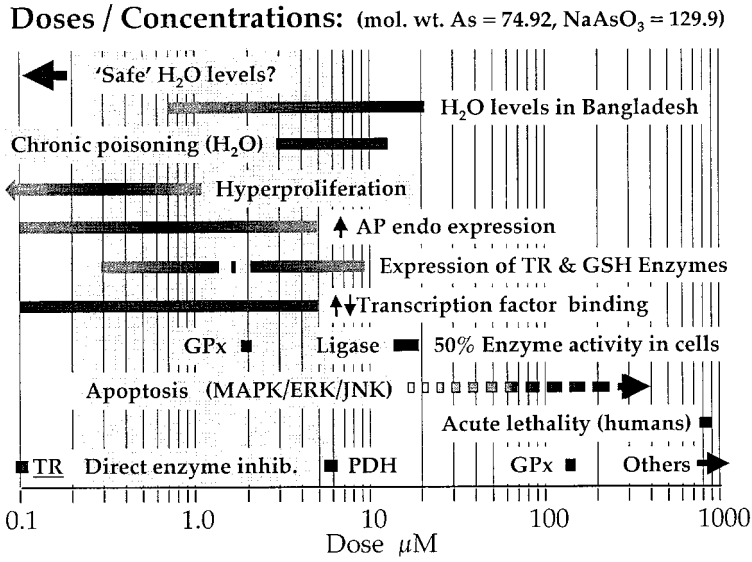

Fig. 6. Relative doses of arsenic to which people are regularly exposed compared to those required to elicit various cellular and molecular responses. The total concentration of As(III) and As(V) in the water of a typical well in Bangladesh ranges from 10 to over 2000 ppb or 0.13 to 27 μM. Chronic arsenicism is most often observed after exposures in the range of 3 to 15 μM (200 to 1000 ppb) As. Hyperproliferation and increased growth of endothelial cells has been observed after a minimum of 24 h exposure of cells to 0.1 to 2 μM As(III). As shown here, maximum induction of BER enzymes occurs after exposure to approximately 1 μM As(III). Down-regulation occurs at higher concentrations and DNA ligase activity is decreased by 50% at concentrations of 8 to 15 μM. Maximum induction of redox genes is seen at up to 20 μM As(III). However GPx is down-regulated at similar doses and 50% GPx activity is observed after 24 h exposure to 2 μM As(III). Transcription factor binding is also altered after exposure of fibroblasts to concentrations of 0.1 to 5 μM As(III). These effects occur at sub-toxic to moderately toxic doses of As(III). Subacute exposure to 8 to 40 μM As(III) for 24 to 48 h leads to 50% toxicity in fibroblasts and keratinocytes. This roughly correlates with amount of As(III) required to directly inhibit pyruvate dehydrogenase (PDH), an enzyme critical for aerobic metabolism. The enzyme activity of TR is sensitive to direct inhibition by as little as 0.1 μM MMA(III) (as shown, bottom left), but is much less sensitive to inorganic As(III). However, very few other enzymes have been identified that are directly inhibited by biologically relevant concentrations of the most prevalent forms of arsenic.

As(III). Up-regulation of the redox enzymes occurs at approximately 10-fold higher doses, from 1 to 10 μM As(III). Reduction of GPx and DNA ligase enzyme activities to levels 50% or below control values is also seen in human cells exposed to 2 or 10 μM As(III), respectively. Most purified enzymes are much less sensitive to direct inhibition by arsenic (Hu et al., 1998; Chouchane and Snow, 2001). However, TR is inhibited by as low as 0.1 μM MMA(III) (Lin et al., 1999) and the pyruvate dehydrogenase complex (PDH) is 50% inhibited by approximately 6 μM As(III) (Hu et al., 1998) or MMA(III) (Petrick et al., 2000). Thus inhibition of these or other acutely sensitive enzymes may be the ultimate cause of arsenic induced cytotoxicity. The critical unknown with regard to arsenic carcinogenesis is how much arsenic in any form actually gets to and is bioavailable to the target tissues, notably the skin and bladder epithelium.

We propose that the biological effects of arsenic are two-fold. This is illustrated in Fig. 7. At low doses, trivalent forms of arsenic such as As(III) and MMA(III) may bind to and activate specific cellular receptors and thereby trigger one or more signal cascades. This results in the altered regulation of genes involved in redox regulation and base

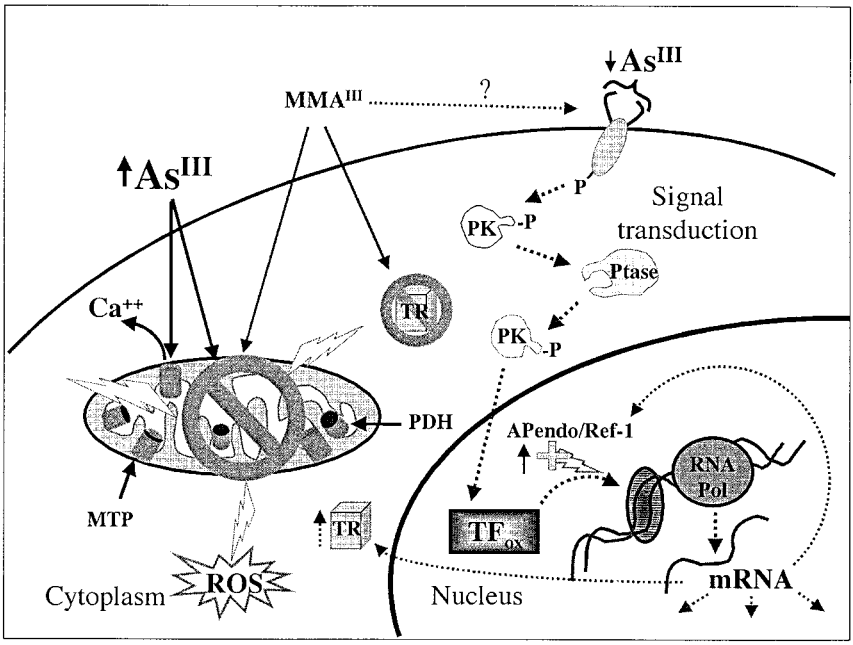

Fig. 7. A speculative model for the dual effect of As(III) leading to a low dose protective response and high dose killing. It is proposed that cell killing is primarily mediated by the inhibition of mitochondrial enzymes such as pyruvate dehydrogenase (PDH) and calcium release due to interaction of As(III) with the redox-regulated mitochondrial transition pore (MTP). Changes in gene expression are possibly the result of triggering one or more signal cascades by As(III) binding to one or more receptor proteins. MMA(III) could act by either of these mechanisms to either induce gene expression or cell killing. It is not known whether inhibition of TR by MMA(III) would trigger either of these pathways or provoke a separate response.

excision repair, including TR and APE/Ref-1. Note, APE/Ref-1 acts as both a repair and redox protein that can itself activate certain transcription factors (TF_{ox}) (Barzilay et al., 1996). At low dose this gene induction provides a protective response against additional oxidative stress. At higher doses BER is down-regulated, possibly resulting in increased oxidative damage and mutagenesis. At even higher doses, the trivalent arsenic, As(III) or MMA(III), binds to and inhibits critical metabolic enzymes such as PDH and the mitochondrial transport protein (MTP). In the case of MMA(III), TR is also inactivated. This leads to disruption of mitochondrial function and the release of reactive oxygen species, intracellular calcium release, and either necrotic or apoptotic cell death.

CONCLUSION

We have found that low doses of As(III) cause a significant change in the expression and activity of a number of proteins and enzymes critically involved in the regulation of BER and cellular redox control. The mechanism of how arsenic causes these changes is not yet fully understood. However, two findings are most relevant for assessment of risk due to exposure to inorganic arsenic: (1) the dose–response is highly non-linear; the As(III)-related gene induction has a clear peak at exposure levels that are less than 50% toxic for human cells; (2) the changes in enzyme activity are due predominantly to changes

in gene expression and are not the result of direct inhibition of the enzymes. These findings need to be incorporated into any reasonable mechanism-based model for risk assessment.

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Arsenic Exposure and Health Effects IV
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The Carcinogenicity of Dimethylarsinic Acid (DMA) in Rats

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ABSTRACT

A major deterrent to investigations of the mechanism of arsenicals as carcinogens has been the lack of an animal model. During the past few years, two groups have demonstrated that dimethylarsinic acid (DMA), administered at high doses in either the diet or drinking water, produces a significant incidence of tumors of the urinary bladder in rats. It was more active in females than in males, in contrast to most bladder carcinogens, and it was without carcinogenic activity for the bladder or other tissues in mice. Additional studies have demonstrated that administration of high doses of DMA to rats following prior administration of known genotoxic carcinogens enhances the production of tumors, not only of the urinary bladder, but also kidney, liver, and thyroid. Like other arsenicals, DMA is considered to be non-DNA-reactive. Although the mechanism of action is unknown, administration of high doses of DMA to rats produces a significant increase in the proliferation of the urothelium. This appears due to significant cytotoxicity and regeneration rather than a direct mitogenic effect. Cytotoxicity of rat and human urothelial cells has been demonstrated in vitro for arsenite and DMA at concentrations approximately equal to those achieved in the urine of rats administered carcinogenic doses of DMA. Although DMA provides a model which might prove useful in further delineating our understanding of the mechanism of action of arsenic carcinogenesis, there are several difficulties in extrapolating from this rat model to humans, notably, the significant differences in metabolism between the rat and humans and the relatively enormous doses utilized in this animal model compared to even high human exposures. Extrapolations therefore need to proceed with caution.

Keywords: rodent carcinogenesis; arsenic; urinary bladder cancer; urothelium

INTRODUCTION

Arsenic is an ubiquitous element, present in various forms in drinking water, food, air, and especially in the earth. Inorganic arsenic has been considered a human carcinogen since the reported association between the ingestion of Fowler's solution and the development of hyperkeratosis and skin cancer (IARC, 1980). Subsequently, it has also been demonstrated as a carcinogen for the lung in association with various mining exposures. More recently, there is evidence from epidemiological studies in areas of the world in which drinking water contains high levels of inorganic arsenic, that it can produce tumors of other internal organs, especially the urinary bladder (Bates et al., 1992; Hopenhayn-Rich et al., 1996).

DMA CARCINOGENESIS IN RATS

A major impediment to furthering our understanding of the mechanisms by which arsenic causes cancer in humans has been the lack of appropriate animal models (IARC, 1980; Huff et al., 2000). Despite its recognition as a human carcinogen, the various forms of arsenic have not been widely tested in animal models, including in standard two-year bioassays. However, recently, administration of relatively high doses of dimethylarsinic acid (DMA) were demonstrated to produce an increased incidence of cancer of the urinary bladder in rats, with females apparently more susceptible than males (van Gemert and Eldan, 1998; Wei et al., 1999). Bladder tumors were induced whether the chemical was administered in the diet (van Gemert and Eldan, 1998) or in the drinking water (Wei et al., 1999). Administration of DMA in drinking water appeared to produce a significantly greater toxicity at comparable doses. Nevertheless, the only tissue showing any incidence of tumors after two years in the rat was the urinary bladder, and the incidences were relatively low. Higher incidences of urothelial hyperplasia were detected.

Following dietary administration, there was hyperplasia observed at a dose of 40 mg/kg and bladder tumors were present at a dose of 100 mg/kg. Doses of 2 and 10 mg/kg of the diet did not produce any effect on the urothelium in the rat (van Gemert and Eldan, 1998). When administered in the drinking water to male rats, bladder tumors and hyperplasia were observed at doses of 50 and 200 mg/l of the drinking water. No increased incidences of urothelial lesions were detected at 12.5 mg/l, compared to controls (Wei et al., 1999).

In contrast, bioassays of DMA administered in the diet and drinking water to mice have been negative with respect to carcinogenicity (van Gemert and Eldan, 1998; Fukushima et al., 2000). DMA was administered in the diet at doses of 8, 40, 200, and 500 mg/kg to of male and female CD-1 mice (van Gemert and Eldan, 1998), and it was administered in the drinking water at doses of 50 and 200 mg/l to male C57BL wild-type mice and p53(+/−) mice for 90 weeks (Fukushima et al., 2000). There was no increased incidence of tumors in these bioassays involving mice. In addition, Tice et al. (2000) administered sodium arsenite to C57BL/6 p53(+/−) male mice for 26 weeks, at a dose of 50 mg/l of the drinking water, and did not detect any increased incidence of tumors. They also found no increase in tumor incidences in mice when DMA was administered in a choline deficient diet or when it was co-administered with p-cresidine or 4-vinyl-1-cyclohexene diepoxide, known bladder and skin carcinogens, respectively.

Yamamoto et al. (1995) administered DMA in the drinking water to male F344 rats after pretreatment with a complex protocol involving the administration of five known carcinogens, including diethylnitrosamine, *N*-methyl-*N*-nitrosourea, 1,2-dimethylhydrazine, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine, and *N*-bis(2-hydroxypropyl) nitrosamine. DMA was administered at doses of 50, 100, 200, or 400 mg/l in the drinking water. The incidences of tumors of the bladder, kidney, liver, and thyroid gland were increased, but only at the highest dose, which was well in excess of a maximally tolerated dose and produced

significant toxicities. Because of the extraordinarily high, toxic doses used in this study, interpretation of the results are difficult.

Administration of DMA in the drinking water at doses of 0, 2, 10, 25, 50, and 100 mg/l for 32 weeks following pre-administration with BBN produced an increased incidence of urinary bladder tumors at doses of 25 mg/l and higher (Wanibuchi et al., 1996). There was a suggestion of a slight effect at 10 mg/l. Again, significant toxicity was seen at the highest dose (100 mg/l), but tumors were seen at lower doses, also. No effects on the urothelium were seen at a dose of 2 mg/l in the drinking water.

DMA administered as 100 mg/l of the drinking water following pretreatment with BBN produced a similar incidence of bladder tumors in NCI-Black-Reiter male rats as in F344 male rats (Li et al., 1999). This strain of rats does not produce or excrete alpha$_{2u}$-globulin in the urine. A lack of difference of results with DMA in the NCI-Black-Reiter rat compared to the F344 rats suggests that this male rat protein is not involved in the mode of action of DMA in bladder carcinogenesis in rats. In general, experiments in mice have been negative. However, DMA has recently been shown to promote skin carcinogenesis in *keratin* (K6)/*ODC* transgenic female mice following initiation with 7,12-dimethylbenz(a)anthracene (DMBA). Treatment with DMA alone had no effect on these mice. However, in strains of mice more commonly utilized for skin initiation–promotion experiments, DMA and other arsenicals, including inorganic arsenic, have been negative (IARC, 1980; Huff et al., 2000; Morikawa et al., 2000; Tice et al., 2000).

MODE OF ACTION FOR DMA CARCINOGENESIS IN THE RAT

Inorganic and organic arsenicals are not DNA reactive chemicals (IARC, 1980; Jacobson-Kram and Montalbano, 1985; U.S. EPA, 1997). Although they have produced positive results in a variety of genotoxicity screens, these involve mechanisms generally associated either with DNA repair, chromosomal aberrations or clastogenicity (IARC, 1980; Jacobson-Kram and Montalbano, 1985). In a few instances, possible mechanisms regarding the cause of these genotoxic alterations have been identified, but for the most part, they have not. It is clear, based on chemical and biochemical data as well as structure–activity relationships, that arsenicals do not and will not react with DNA (IARC, 1980; Jacobson-Kram and Montalbano, 1985; U.S. EPA, 1997). However, arsenicals, particularly As(III) species are well known to interact with protein sulphydryl groups, including some proteins that are known to be involved with DNA repair, microtubule structure and other proteins that are involved with the integrity of DNA and chromosome structure (IARC, 1980; Wang and Rossman, 1996; Rossman, 1998).

In the human, arsenic-induced skin carcinogenesis appears to involve hyperkeratosis associated with epidermal hyperplasia (IARC, 1980; Schwartz, 1997). It is unclear whether this is due to a direct mitogenic effect of inorganic arsenic on the epidermis or if it is due to toxicity and regeneration (Germolec et al., 1998).

Since DMA is not directly DNA reactive, carcinogenesis in the rat bladder secondary to administration of high doses of DMA is likely caused by increased proliferation, either due to increased cell births or decreased cell deaths. There is no evidence that arsenicals have a direct effect on apoptosis or differentiation of the bladder epithelium of the rat. Therefore, it is most likely that the increased cell proliferation is due to an increase in cell births. This could be due to direct mitogenesis or toxicity followed by regeneration.

We demonstrated that DMA at 40 and 100 mg/kg in the diet produced cytotoxicity and increased proliferation of the bladder epithelium; at lower doses this was not observed (Arnold et al., 1999). Similar effects have been seen by Fukushima and colleagues utilizing DMA in the drinking water at comparable overall doses (Wanibuchi et al., 1996). The hyperplasia that is produced is reversible upon discontinuation of DMA administration (Arnold et al., 1999). We demonstrated that the cytotoxicity was not due to the formation

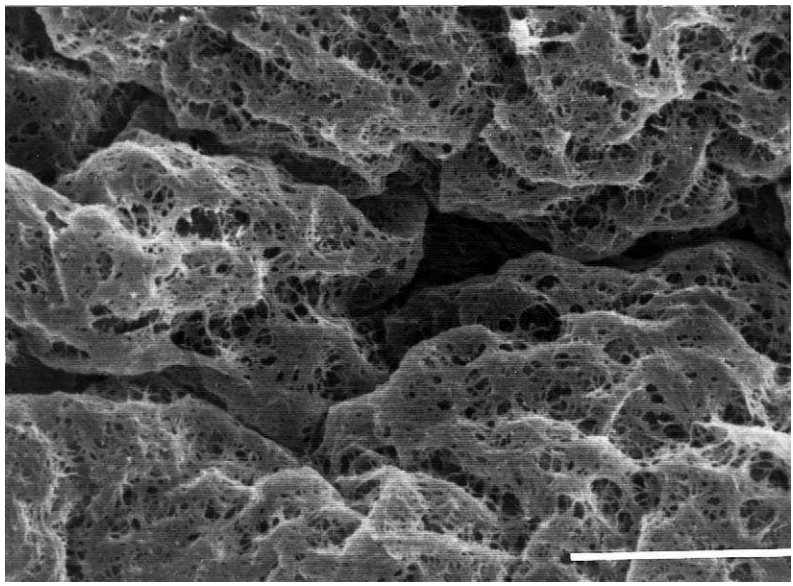

Fig. 1. Cellular cytotoxicity of the bladder epithelium 6 h after feeding of 100 mg/kg DMA to a female rat (bar = 10 μm).

of urinary solids in the rat following DMA administration, either precipitate, microcrystalluria, or calculus formation (Arnold et al., 1999). There was some evidence that increased urinary calcium excretion might contribute to the toxicity or proliferation (Arnold et al., 1999). This did not answer whether the cell proliferation occurred first or whether it was toxicity with consequent regeneration.

In recent experiments in our laboratory, we have demonstrated that DMA, administered as 100 mg/kg of the diet, produces a cytotoxic effect on the urothelium within 6 h of administering the compound (Fig. 1), whereas the increase in cell proliferation occurs after three days of administration (Arnold et al., 2000). The increase in labeling index appears to increase through two weeks of administration and then decreases by ten weeks of administration, although it is still well above control levels. These studies clearly demonstrate that DMA administration produces a cytotoxic response in the urothelium with consequent regeneration (Fig. 2), rather than direct mitogenesis. The urinary calcium changes following DMA administration were not seen at the earlier time points when cytotoxicity and regeneration had already occurred. Whether urinary calcium abnormalities contribute to the continued proliferative stimulus to the bladder epithelium is unknown, but it is clear that it is not essential to its induction.

The exact mechanism by which DMA administration produces cytotoxicity on the bladder epithelium is unknown. We have demonstrated that sodium arsenite is cytotoxic to rat urothelium in culture at doses of approximately 0.5 μM (Yamamoto et al., 2000). In the same experiments, sodium arsenate produced cytotoxicity at concentrations of approximately 5 μM whereas DMA and monomethylarsonous acid (MMA) were cytotoxic at concentrations of 0.2 mM and 1 mM, respectively. Wanibuchi et al. (1996) and Yoshida et al. (1997) reported that DMA administered in the drinking water resulted in urinary concentrations of approximately μM levels of arsenite and mM concentrations of DMA. The concentrations of arsenate and MMA were significantly less than 1 μM. Assuming that the toxicity to the urothelium following DMA administration occurs via urinary exposures, the in vitro data and the urinary concentrations following DMA administration to rats suggest

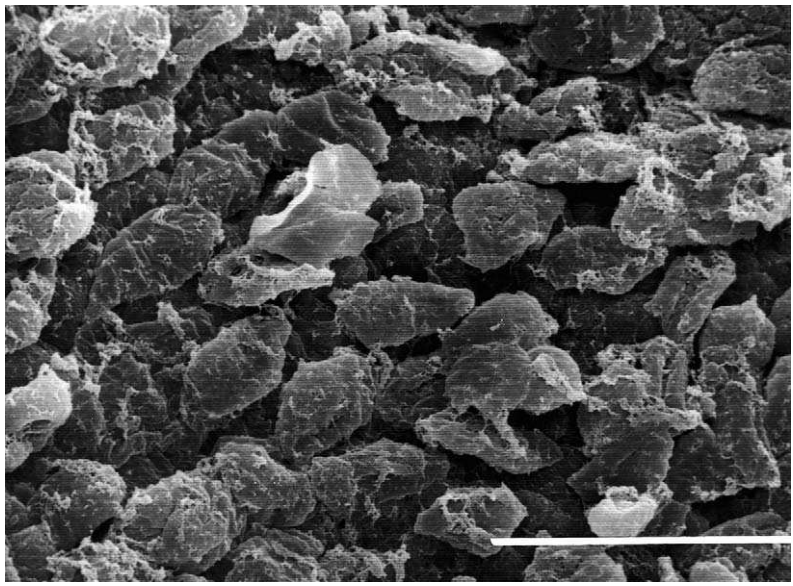

Fig. 2. Extensive cellular necrosis and exfoliation of the female rat urothelium after 7 days of DMA administered as 100 mg/kg in the diet (bar = 0.1 mm).

that toxicity is most likely due to arsenite, but possibly also due to DMA. It is unlikely that arsenate or MMA are responsible, considering their extremely low concentrations in the urine. However, the effects of MMA(III) have not been evaluated either in urine or in urothelial culture systems.

IMPLICATIONS FOR HUMAN RISKS

Two laboratories have confirmed the bladder carcinogenicity of DMA in the rat (van Gemert and Eldan, 1998; Wei et al., 1999). However, there are at least two major difficulties in extrapolating these results to possible human risk. First, the doses required to produce the effect in the rat are extremely high in comparison to human exposures (IARC, 1980). Clearly, considerably more research is required to determine the dose response effects in the rat and to determine what the implications are with respect to human exposures. Although the exact mechanism of carcinogenesis of arsenicals in general, including DMA, is not known, there is general agreement that the dose response is non-linear since none of the arsenicals are DNA reactive (Abernathy et al., 1996; U.S. EPA, 1997). In addition, information regarding the lack of carcinogenicity of DMA in the mouse and determination of its effects in other species needs to be explained.

Of greater difficulty in extrapolating the results from the rat to humans are the extraordinary differences in the metabolism of arsenicals in rodents compared to humans, especially for the rat. Aposhian (1997) has indicated that rodent metabolism of arsenicals is quite different from humans, and this is especially true for the rat. This is largely because of the unique processing of arsenicals by the rat, which includes uptake and storage of the arsenicals in red blood cells so that the chemical's half life in the rat corresponds to the half life of red blood cells. This is in striking contrast to the rather rapid excretion and lack of accumulation of arsenicals in humans. Also, the extent of methylation and demethylation varies considerably between species.

In summary, DMA produces bladder cancer in rats when administered at relatively high doses in the diet or in the drinking water. It appears to be due to the induction of cytotoxicity of the urothelium with consequent regeneration. Extrapolation of these results to humans must be made with considerable caution since the animal studies involve extraordinarily high doses compared to human exposures and because of the significant differences in metabolism between the rat and humans.

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Arsenic Exposure and Health Effects IV
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Human Cell Models for Arsenic Carcinogenicity and Toxicity: Transformation and Genetic Susceptibility

Toby G. Rossman, Melissa A. Visalli, Ahmed N. Uddin, Yu Hu

ABSTRACT

Humans are more sensitive to arsenic compounds than are rodents by at least an order of magnitude. This is also true on the cellular level. The lack of an arsenite-inducible tolerance mechanism in human cells may partly account for their sensitivity. In addition, inter-individual variability in sensitivity to arsenic is well known. It is therefore important to study the effects of arsenic compounds on human cells. We have found that extremely low and completely nontoxic concentrations of arsenite (0.1 nM) can transform human osteosarcoma (HOS) cells to anchorage-independence, a marker for tumorigenicity. The transformation is seen only after extensive growth (~8 weeks) in the presence of arsenite. HOS transformation will be an important system with which to study the mechanism of arsenite carcinogenicity. We have also started a study of genetic diversity by measuring the sensitivities of 14 lymphoblast lines derived from normal human donors. We show that growth inhibition by arsenite differs significantly in different lymphoblast lines, and that the differences are not related to the growth rates of the lymphoblasts. By comparing arsenite metabolism, uptake, efflux, and specific gene expression between the most sensitive and most resistant lines, we may be able to identify genes of importance in arsenic sensitivity.

Keywords: transformation; growth rate; anchorage independence; genetic variability; arsenic

INTRODUCTION

Arsenic is a common environmental contaminant of our air, water and food. Sources of arsenic contamination are both natural and man-made. Epidemiological studies clearly demonstrate that inorganic arsenic compounds are human skin, bladder, and lung (and possibly liver and kidney) carcinogens, but they are not reproducibly tumorigenic in animal bioassays (Léonard, 1991; Rossman, 1998). Besides cancers, chronic arsenic exposure causes hyperkeratosis, pigmentation changes, and effects on the circulation, liver and nervous systems (National Research Council, 1999). Human exposure to arsenic compounds is widespread, and results from both natural and man-made sources. Among all established human carcinogens, arsenic is the only one for which there is no good animal model. Because of this, arsenic compounds are the only compounds that IARC considers to have sufficient evidence for human carcinogenicity, but inadequate evidence for animal carcinogenicity (International Agency for Research on Cancer, 1980). The increase in cancer risk observed in epidemiological studies is attributed mainly to the presence of inorganic trivalent arsenic (International Agency for Research on Cancer, 1980; Landrigan, 1992).

Humans appear to be more sensitive to the toxic effects of arsenic compounds, compared with rodents. For example, the LD_{50} of arsenic trioxide (96 h, oral) is 25.8 and 39.4 mg/kg for C_3H and Swiss mice, respectively (Léonard, 1991). Although there is no way to calculate the human LD_{50}, it is of interest that the lethal dose of arsenic trioxide is reported to be 70–180 mg (or about 1–2.6 mg/kg) (Léonard, 1991). This means that the LD_{50} could be lower, assuming that the exposed people had average sensitivities. This difference between humans and rodents also exists on the cellular level. Lee and coworkers were the first to point out that human fibroblasts are more sensitive to arsenite than Chinese hamster CHO cells (Lee et al., 1989). This laboratory found that Chinese hamster V79 cells exhibit the phenomenon of inducible arsenite tolerance (Wang and Rossman, 1993). When these cells are treated with subtoxic concentrations of arsenite or antimonite, a tolerance mechanism is induced which allows these cells to survive otherwise toxic concentrations of arsenite, arsenate, or antimonite. Maximum tolerance is reached by 6–8 h after treatment, and requires de novo mRNA and protein synthesis (Wang et al., 1994). When arsenite-induced cells are grown in the absence of arsenite for 3 weeks and re-tested, the level of resistance is the same as that in non-adapted cells. This tolerance mechanism is different from the heat shock response (Wang et al., 1994) and is not caused by differences in levels of GSH or GSH transferase (T.G. Rossman, Z. Wang and E.I. Goncharova, unpubl. data).

We also showed that all human cell lines tested are considerably more sensitive to arsenite compared with Chinese hamster cells (Table 1) and no human cells tested show arsenite-inducible tolerance to arsenite (Rossman et al., 1997). We concluded that one factor contributing to the greater arsenite sensitivity of human cells compared with hamster cells is the inability of human cells to induce one or more protective proteins in response to arsenite. This suggests that arsenite's effects on signal transduction pathways resulting in de novo protein synthesis may differ between rodent and human cells. Because of these differences, it is important to develop human cell models to study arsenic toxicity and carcinogenicity.

Not every individual who drinks arsenic-contaminated water shows clinical signs of toxicity. For example, in Bangladesh and West Bengal, skin lesions were found in 24.47% and 15.02% of people from affected villages, respectively (Chowdhury et al., 2000). Recent advances in human genetic toxicology research has also revealed the existence of heterogeneity in the human response to arsenic compounds. An evaluation of arsenite-induced aneuploidy in peripheral blood lymphocytes from human donors show quite large inter-individual variation (Vega et al., 1995). For example, at 0.01 μM arsenite, the heteroploidy frequency in lymphocytes from different individuals varied from 12.5% to 52%. Donors who were most sensitive to arsenite-induced aneuploidy were also most sensit-

TABLE 1

Sensitivity of human and Chinese hamster cell lines to sodium arsenite (clonal assay)

Cell line	Type	ID_{50} * (μM)	Reference
Chinese hamster			
V79	Chinese hamster lung fibroblast	12.5	Rossman et al. (1997)
CHO	Chinese hamster ovary	25.0	Rossman et al. (1997)
Human			
CRL1295	Normal diploid fibroblast	0.6	Rossman et al. (1997)
WI38	Normal diploid fibroblast	1.9	Vogt and Rossman (2001)
HeLa	Cervical carcinoma	1.6	Rossman et al. (1997)
AG06	SV40-transformed keratinocyte	0.2	Rossman et al. (1997)
HOS	Human osteosarcoma	0.5	This article (Fig. 1)
HTB139	Meduloblastoma	1.4	Rossman et al. (1997)
2008	Ovarian carcinoma	2.0	Naredi et al. (1995)

ID_{50} = concentration of sodium arsenite which results in 50% decrease in a clonal cytotoxicity assay (continuous arsenite exposure).

ive to arsenite-induced mitotic arrest and chromosome aberrations. Large inter-individual variations were obtained by other laboratories studying arsenite-induced chromosome aberrations (Wiencke and Yager, 1992) and sister chromosome exchanges (SCEs) (Crossen, 1983) in cells from different donors.

Further evidence for heterogeneity in humans with respect to arsenic susceptibility, although of a more indirect nature, was obtained in epidemiological studies. Blackfoot disease, a unique peripheral vascular disease, results from long term exposure to arsenic in drinking water in some areas of Taiwan and Mexico. Subjects with Blackfoot disease have a significantly higher risk of developing cancers compared to others drinking the same water who do not develop Blackfoot disease (Chiou et al., 1995). This is true even after controlling for the cumulative arsenic exposure, and points to inter-individual variations in susceptibility to arsenic toxicity and carcinogenicity.

Comparison of arsenite-induced SCE frequency in primary lymphocyte cultures from normal individuals also showed substantial variability, (Rasmussen and Menzel, 1997). Lymphoblasts are virally immortalized lymphocytes which retain most of the properties of lymphocytes. When data from the primary lymphocytes was compared with that obtained with the lymphoblasts derived from the same individual, the slopes of the dose–response curves for arsenite-induced SCEs were similar. Individual lymphoblastoid cell lines retained their arsenite sensitivity after cryopreservation and subsequent revival. This study establishes a rationale for using human lymphoblast lines in studying human heterogeneity, and allows us to take advantage of the numerous human lymphoblast lines available in cell banks.

METHODOLOGY

Sodium Arsenite

Stock solutions of 25 mM sodium arsenite (Sigma Chemical Co., St. Louis, MO) in sterile distilled water were prepared immediately before use, and aliquots were added to the cultures to give the desired final concentrations.

HOS Cell Cultures and Anchorage-Independent Growth

HOS (Human osteosarcoma cells TE85, obtained from American Type Culture Collection, Rockville, MD) were cultured in α-MEM (Gibco BRL, Grand Island, NY) containing 10%

fetal bovine serum (Gibco BRL, Grand Island, NY). Cultures were maintained in 5% CO_2 at 37°C. Sodium arsenite at various concentrations was added to the medium and growth took place for a period of eight weeks. Colony formation in soft agar was carried out according to the method of Rani and Kumar (1992) All assays were carried out in triplicate.

Clonal Survival Assays

Cells were seeded at a density of 500 cells/60 mm dish with arsenite being added after cell attachment. After 7 days' growth, colonies were fixed in methanol, stained in 0.5% crystal violet/50% methanol and counted. All assays were carried out in triplicate.

Lymphoblast Cultures

The 14 lymphoblast cell lines used for this study were purchased from Coriell (Cell Repositories, Camden, NJ). They were established by Epstein–Bar virus transformation of peripheral blood mononuclear cells of clinically unaffected Caucasian females. Cryopreserved cells were thawed, washed and grown in RPMI 1640 medium (Gibco BRL) containing 1% L-glutamine and 15% heat inactivated fetal bovine serum in a 37°C incubator gassed with 5% CO_2. To assay effects of arsenite on growth, exponentially growing lymphoblast cells were counted and replated at 1×10^6 cells in 5 ml medium in 6-well tissue culture plates. Sodium arsenite was added to the cultures to give the final concentrations indicated. The cells were maintained up to 10–12 days without changing medium. They grew in suspension culture with cells clumped in loose aggregates. On alternate days, the aggregates were dissociated by gently agitating the culture with a pipette and the number of viable cells in each well was determined using a hemocytometer and trypan blue stain, counting trypan blue negative cells. Each experiment was repeated at least once.

RESULTS

An Assay for Arsenic Transformation of Human Cells

Fig. 1 shows the sensitivity of HOS cells to sodium arsenite. As is typical for other human cells (Table 1), these cells show an ID_{50} of about 0.5 μM for clonal survival. However, when grown in monolayer culture, the cells are less sensitive to growth inhibition by arsenite. We therefore chose to use concentrations of 0.1–0.3 μM arsenite in the first experiment to determine if arsenite will transform HOS cells to anchorage-independence. HOS cells only rarely transform to anchorage-independence spontaneously. However, cells grown in the presence of 0.1–0.3 μM arsenite showed significant transformation at all concentrations (Table 2). No transformation occurred after 2 weeks, and the data in Table 2 shows results after 8 weeks. There was no clear relationship of transformation rate to dose. To determine whether there is a threshold concentration for transformation, a second experiment was performed at much lower concentrations. Surprisingly, transformation occurred even at 0.0001 μM (0.1 nM). Experiments are underway to determine the minimum concentration needed for this transformation event, and the molecular mechanism responsible.

Genetic Variability in Sensitivity to Arsenite

To determine whether normal human lymphoblasts show variability toward arsenite toxicity, 14 normal human lymphoblast lines, derived from clinically unaffected female subjects were obtained. The subjects' ages ranged from 10 to 53, with a mean age of 29.7. Fig. 2 shows the effects of various concentrations of arsenite on the growth of a relatively resistant and relatively sensitive lymphoblast line, respectively. The resistant line (Fig. 2A) is able to grow in concentrations of arsenite up to 0.5 μM, although the growth rate is decreased in a dose-dependent manner. The sensitive line (Fig. 2B) not only fails to grow in concentrations of arsenite 0.2 μM and above, but there are decreases in the cell counts

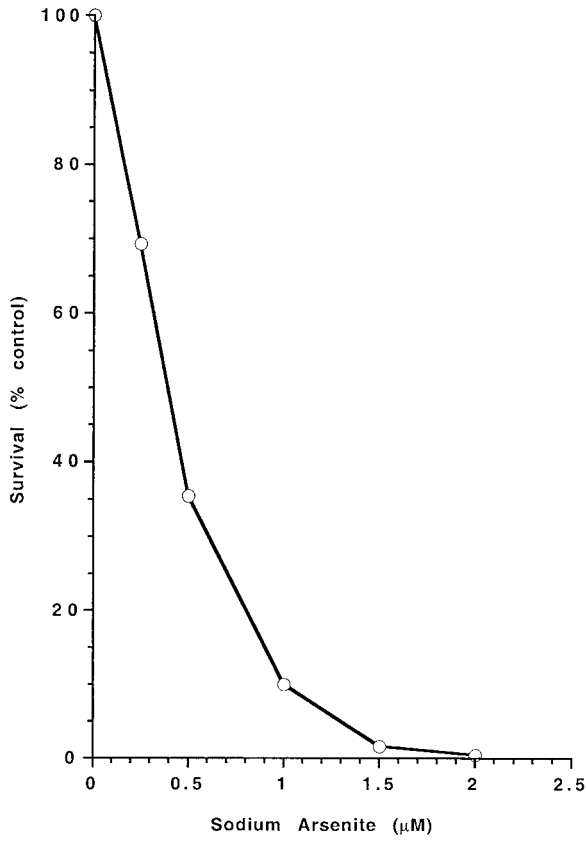

Fig. 1. Sensitivity of HOS cells to sodium arsenite. HOS cells were plated for clonal growth as described in the Methodology section and exposure to arsenite was continuous.

at these concentrations, indicating cell death. Fig. 3 shows a histogram of the sensitivities of the 14 lymphoblast lines to 0.2 μM arsenite on the 6th day of growth. There are not enough data at this point to determine whether the distribution is unimodal or bimodal, but it is clear that variability exists, since the range of effects is almost 7-fold. We intend to perform similar assays on a total of 60 lymphoblast lines, from both male and female donors, and from different racial groups.

We also noted that the lymphoblast lines vary with regard to their growth rates (e.g., controls in Fig. 2). It is conceivable that slow-growing cells would show increased sensitivity to growth inhibition by arsenite. We therefore plotted the growth rate (number of cells in the culture at day 6, starting at an inoculum of 10^6 cells) against the effects of 0.2 μM arsenite on the growth rate on that day. As is clear from Fig. 4, there is no relationship between growth rate and arsenite sensitivity.

DISCUSSION

The non-tumorigenic human osteoblast-like HOS cells do not exhibit anchorage-independent growth, but can be transformed to anchorage-independence (and tumorigenicity) by a variety of carcinogens (Rhim et al., 1975; Lin and Costa, 1994; Miller et al., 1998). This is the first report of transformation of HOS by arsenite. Table 3 lists previous studies on cell transformation by arsenite. There has been only one report of transformation of human

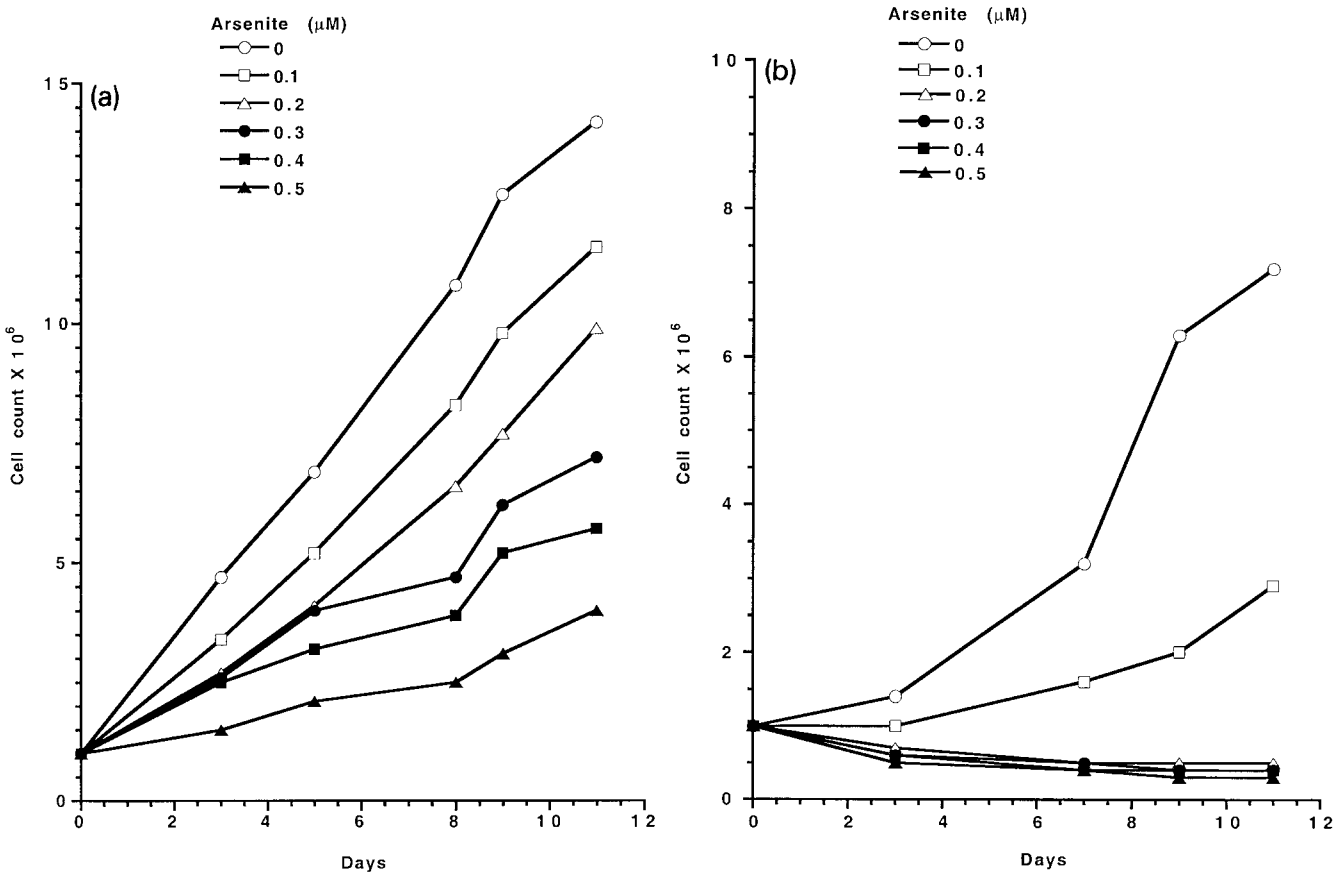

Fig. 2. Inhibition of growth of two human lymphoblast lines by sodium arsenite. Lymphoblasts were grown and assayed as described in the Methodology section. (A) An example of a resistant lymphoblast line. (B) An example of a sensitive lymphoblast line.

TABLE 2

Transformation of HOS cells to anchorage-independence by arsenite

Sodium arsenite, μM	Transformation frequency, $\times 10^{-4}$
Experiment 1	
0	2
0.1	58
0.2	55
0.3	64
Experiment 2	
0	1
0.01	68
0.001	56
0.0001	32

Treatment time was 8 weeks. No transformation by arsenite was seen after a 2-week treatment.

TABLE 3

Transformation of mammalian cells by arsenite

System	Endpoint	Smallest effective concentration	Reference
Syrian hamster embryo cells	enhancement of viral transform.	100 mM	Casto et al., 1979
Syrian hamster embryo cells	morphological transform.	0.7 mM	Lee et al., 1985
Mouse BALB/3T3	morphological transform.	3 mM	Saffiotti and Bertolero, 1989
Mouse BALB/3T3	morphological transform.	10 mM	Sabbioni et al., 1991
Mouse C3H/10T1/2	enhancement of viral transform.	5 mM	Kowalski et al., 1996
Rat (liver) TRL1215	morphological transform.	0.125 μM (18 weeks)	Zhao et al., 1997
Human diploid fibroblasts	anchorage independence	1 μM	Biederman and Landolph, 1987
Mouse JB6 CI 41 (epidermal)	anchorage independence	25 μM	Huang et al., 1999

cells by arsenite. Anchorage-independent growth but no focus formation or immortality was induced by 1 μM arsenite in diploid human fibroblasts (Biederman and Landolph, 1987). None of the studies listed in Table 3 determined the minimum dose needed to transform cells by arsenite (and neither have we). Only one study used long-term exposures (Zhao et al., 1997) and found transformation at submicromolar concentrations.

Growth in soft agar is the most consistent criterion for the malignant state, correlating more strictly than other criteria with the ability of a cell to grow as a tumor when injected into a suitable host (Shin et al., 1975). Anchorage-independent activation of G_1 cyclin-dependent kinases can mediate the anchorage-independent growth phenotype (Yang et al., 1998). HOS cells transformed by nickel compounds had hypophosphorylation of the tumor suppressor protein Rb (Lin et al., 1994). In a rat liver-derived system, transformation by arsenite was associated with global DNA hypomethylation, decreased DNA methyltransferase activity and activation (overexpression) of the proto-oncogene c-*myc* (Zhao et al., 1997). Arsenite-induced transformation of JB6 cells was mediated by phosphorylation of Erk (Huang et al., 1999). It will be of interest to see whether HOS cells transformed by arsenite show any of these alterations.

Biomethylation of arsenic has long been considered the major detoxication pathway. However, it now appears likely that the trivalent methylated metabolites may be important for some of the adverse effects of arsenic (Petrick et al., 2000; Styblo et al., 2000). HOS cells are able to methylate arsenite, but at a rate one order of magnitude slower than human

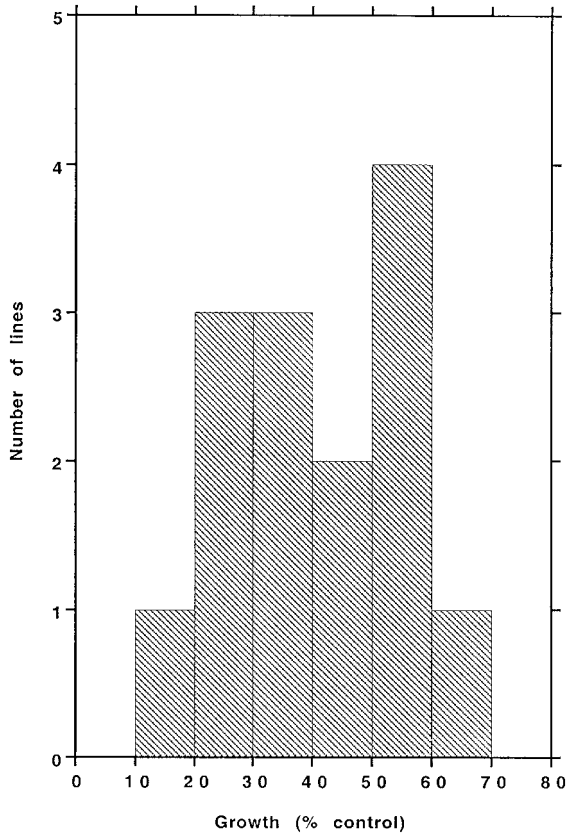

Fig. 3. Histogram showing the sensitivities of 14 lymphoblast lines to growth inhibition by 0.2 μM sodium arsenite.

hepatocytes (M. Styblo, pers. commun., 2000). Experiments are underway to determine if trivalent methylated species can transform HOS cells.

Inter-individual variations in the ability of humans to methylate arsenic have been reported (reviewed in Vahter, 2000). However, this cannot account for the variability in sensitivity to arsenite seen in the lymphoblast lines, because no methylated species were detected in three lymphoblast lines with differing sensitivities (M. Styblo, pers. commun., 2000). Nor were there any differences in cellular retention of arsenic (M. Styblo, pers. commun., 2000). What, then, could be the cause of the variability? A number of possibilities exist. These include: (1) variability in the structure or abundance of target molecules (although these are presently unknown, we have cloned two genes whose up-regulation results in increased resistance to arsenite (Rossman and Wang, 1999) suggesting that their products may be possible targets); (2) genetic variability in the structure or abundance of protective mechanisms such as efflux pumps, antioxidants or heme oxygenase (Lo et al., 1992; Lee and Ho, 1994; Wang et al., 1996); or (3) alterations in signaling pathways that result in growth control or apoptosis.

CONCLUSIONS

Two human cell systems which should be useful for studying the mechanisms of arsenic toxicity and carcinogenicity have been described: (1) human osteosarcoma (HOS) cells,

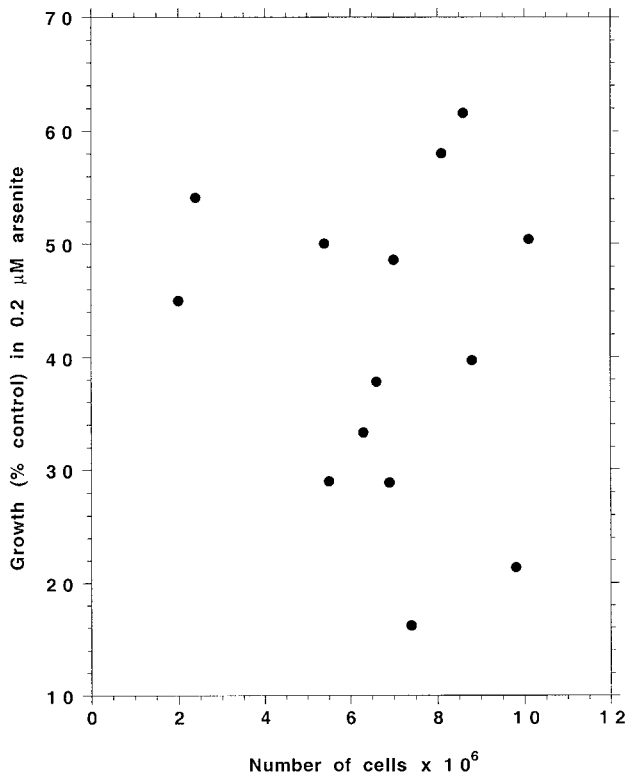

Fig. 4. Lack of relationship between growth rates and arsenite sensitivities among the lymphoblast lines.

which can be transformed to anchorage-independence by extremely low (0.1 nM–0.1 μM) concentrations of arsenite; (2) lymphoblastoid cells, derived from clinically normal individuals, which can be used to study genetic variability to the toxic effects of arsenic compounds.

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Arsenic Exposure and Health Effects IV
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Research Requirements for a Biologically Realistic Cancer Risk Assessment for Arsenic

Harvey Clewell

ABSTRACT

The Environmental Protection Agency has proposed reducing the current Maximum Contaminant Level (MCL) for arsenic in drinking water in the U.S., based on cancer risk estimates obtained from a highly exposed Taiwanese population using a linear dose–response model. Currently proposed modes of action for the carcinogenicity of arsenic are generally consistent with the expectation of a nonlinear dose–response; however, further experimental data are needed to provide the basis for accurately determining a quantitative nonlinear dose–response that could serve as an alternative to the linear default. Specifically, a biologically based risk assessment for arsenic will require research to (1) discriminate between competing hypotheses on the mode(s) of action for arsenic carcinogenicity, (2) determine the in vitro dose–response for the critical cellular effect(s) of arsenic, and (3) estimate the tissue concentrations of the relevant arsenic species that result from human exposures. In addition, research is needed to identify and quantify the impacts of factors that modulate individual sensitivity to the carcinogenicity of arsenic. Pharmacokinetic and biologically based dose–response modeling can play an important role in integrating these various data into a quantitative framework suitable for conducting a scientifically defensible risk assessment for the carcinogenicity of arsenic. These same modeling approaches can also be used in the context of Monte Carlo analysis to evaluate the impact of inter-individual variability on the aggregate dose–response for the population, in order to ensure the protection of sensitive individuals.

Keywords: arsenic; cancer; risk assessment; maximum contaminant level

INTRODUCTION

The US Environmental Protection Agency (US EPA) has proposed a Maximum Contaminant Level (MCL) for arsenic in drinking water of 0.01 mg/l (US EPA, 2001). This MCL was derived on the basis of the estimated dose–response for bladder and lung cancer in a population in Taiwan chronically exposed to concentrations of arsenic in drinking water ranging as high as 1.75 mg/l (NRC, 1999; Morales et al., 2000). These dose–response calculations were performed under the standard default assumption of linearity, despite mounting evidence that the mode of action for the carcinogenicity of arsenic is nonlinear (Clewell et al., 1998; NRC, 1999). The proposed US EPA cancer guidelines (US EPA, 1996) provide for the possibility of a departure from the linear dose–response default in the case of carcinogens with a nonlinear mode of action. However, the US EPA felt unable to depart from the linear default in the case of arsenic, due to the lack of definitive data on a specific nonlinear mode of action for its carcinogenicity (US EPA, 2001). Unfortunately, the result of the use of a linear dose–response calculation is a highly conservative drinking water standard, which may entail very significant costs to many local communities in the United States (US EPA, 2001).

In February, 2001, the US Environmental Protection Agency (US EPA, 2001) proposed a Maximum Contaminant Level (MCL) for arsenic in drinking water of 0.01 mg/l. This MCL was selected from several options (0.003, 0.005, 0.01, and 0.02 mg/l) on the basis of a cost–benefit analysis in which the benefits were estimated using information on the dose–response for bladder and lung cancer in a population in Taiwan chronically exposed to arsenic in drinking water (NRC, 1999; Morales et al., 2000). In particular, the agency used the results of the cost–benefit analysis to justify departure from the Practical Quantitation Limit (PQL) of 0.003 mg/l because the costs of implementation would exceed the benefits derived. The ratio of benefits (cancers avoided) to costs (of treatment) at the various MCL options (US EPA, 2001) are shown in Table 1.

In this cost–benefit analysis the agency used 'lower bound' risk estimates that the agency derived by adjusting the results of Morales et al. (2000) to account for arsenic in the food ingested by the Taiwanese population. Specifically, the adjustment took into consideration (a) 1 l drinking water used to re-hydrate food, and (b) 0.05 mg/day arsenic in the food itself. Table 2 compares the US EPA (2001) 'upper bound' estimates, using the risk estimates from Morales et al. (2000) for males and females, with their 'lower bound' estimates, for males only, adjusted for arsenic from food.

From a comparison of Table 1 and Table 2, it can be seen that if the upper bound risk estimates were used in the cost–benefit analysis, the estimated benefits (cancers avoided)

TABLE 1

Benefit/cost ratios at MCL options

MCL option (mg/l):	0.003	0.005	0.01	0.02
Benefit/cost ratio:	0.6	0.8	1.0	1.0

TABLE 2

Comparison of US EPA 'upper bound' and 'lower bound' risk estimates for bladder and lung cancer for U.S. populations exposed at or above MCL options, after treatment

MCL option (mg/l):	0.003	0.005	0.01	0.02
Upper bound risk ($\times 10^{-4}$):	0.93–1.25	1.63–2.02	2.41–2.99	3.07–3.85
Lower bound risk ($\times 10^{-4}$):	0.11–0.13	0.27–0.32	0.63–0.76	1.1–1.35

TABLE 3

Approximate reduction in risk estimates for arsenic in drinking water due to US EPA adjustments for arsenic from food

MCL option (mg/l)	Reduction in risk estimate
0.003	4.7
0.005	3.2
0.01	2.1
0.02	1.6

would exceed the costs (of treatment) at all of the MCL options, including the PQL of 0.003 mg/l. The differences between the upper and lower bound risk estimates result from several factors. However, as shown in Table 3, the adjustment for arsenic in food was the major factor in decreasing the risk estimates from Morales et al. (2000) for the lower MCL options. In particular, the food adjustment lowered the risks at the PQL by nearly a factor of 5.

In performing this adjustment for arsenic in food, US EPA (2001) assumed that the entire study population was exposed to 0.05 mg/day arsenic in food, regardless of their exposure to arsenic in drinking water. However, if it is assumed that the entire cohort was exposed to a uniform 50 μg/day arsenic in food, then food arsenic exposure would be independent of well water concentration and would contribute to the baseline (zero well water concentration) risk estimated for the population. Thus in the relative risk approach used by Morales et al. (2000), the ingestion of arsenic in food was implicitly considered through its contribution to the background rate. Therefore it was not appropriate to adjust the results of this analysis for a contribution from arsenic in food. From a comparison of Table 1 and Table 3, it would appear that if the food adjustments were not performed, but the other assumptions for the lower bound risk estimates were retained, the benefit/cost ratios would still be greater than unity at all of the MCL options, in which case the agency would be required to set the MCL at the PQL of 0.003 mg/l.

It is also important to note that the upper bound risk estimates shown in Table 2 would all be considered unacceptable based on past agency practice of accepting risks of no more than 1 in 10,000. Moreover, if these linear risk estimates were accurate, they would only represent a fraction of the total risk from arsenic, due to exposure from sources other than drinking water. Recent analyses estimate daily intakes of arsenic in food in the U.S. to be on the order of 0.05 mg/day, of which about 20–25%, or about 0.01 mg/day, is inorganic arsenic (Borum and Abernathy, 1994). Assuming similar bioavailability of arsenic from food and water, the risks associated with exposure to inorganic arsenic in the food can be estimated from the results obtained for arsenic in drinking water. As shown in Table 2, the upper bound estimate for the lifetime excess risk of bladder and lung cancer from arsenic in drinking water is approximately 2 in 1000 for an arsenic concentration of 0.005 mg/l. Assuming the consumption of 1 to 2 l of drinking water per day, this concentration would correspond to a daily inorganic arsenic intake from drinking water on the order of 0.005 to 0.01 mg/day. Therefore, for an average dietary exposure to inorganic arsenic in food of 0.01 mg/day, the linear risk estimate associated with dietary arsenic alone would be greater than 1 in 10,000, effectively doubling the risks shown in Table 2 for drinking water exposure alone.

DISCUSSION

The risk estimates for exposure to arsenic in U.S. drinking water projected by the US EPA on the basis of the Morales et al. (2000) analysis certainly should provide cause for concern.

However, mechanistic and empirical evidence of a nonlinear mode of action for arsenic carcinogenicity provides a strong basis for belief that the actual risk at these options may be substantially less than the linear estimates (Abernathy et al., 1996; Clewell et al., 1998). While there is currently no completely satisfying description of a specific mode of action for arsenic carcinogenicity, it almost certainly involves the binding of trivalent inorganic arsenic to key cellular proteins involved in cell cycle control (Clewell et al., 1998). In general, interactions of trivalent inorganic arsenic with cellular proteins begin to occur at concentrations on the order of 0.1 micromolar. The dose–response for these effects is highly nonlinear, since there are essentially no effects until a critical fraction of the affected protein has been inhibited. The cellular concentrations at which this 'threshold' occurs have been suggested to be associated with drinking water exposures on the order of the current MCL (Dr. Daniel Menzel, presentation at the SEGH Fourth International Conference on Arsenic Exposure and Health Effects, San Diego, June, 2000). However, there are not yet any published data on cellular concentrations of trivalent arsenic in humans exposed to high concentrations of arsenic in drinking water to verify this suggestion. Indeed, it is entirely possible that even if the mode of action for the carcinogenicity of arsenic is highly nonlinear, the nonlinearity could occur at concentrations well below those of concern for environmental exposure, and the dose–response in the range of the current and proposed MCLs could actually be linear. Nevertheless, preliminary in vitro data with human cells (Snow et al., 2001) are highly suggestive of a nonlinearity in the range of the current and proposed MCLs.

If one is willing to assume that a nonlinearity in the dose–response for arsenic carcinogenicity occurs in the vicinity of the current MCL, then the actual risks in the range of the proposed MCLs (0.003–0.02 mg/l) could be much lower than the linear estimates, and even as low as zero. Based on the assumption that binding to a key cellular control protein underlies the mode of action, the nonlinearity in the cancer dose–response curve would perhaps extend over approximately an order of magnitude, with risks similar to those estimated from the Taiwanese study at the high end, and essentially zero risk at the low end. Due to variability in the sensitivity of individuals across a population, it can be expected that average population risks would decrease in a lognormal (probit) fashion across the region of nonlinearity. Fig. 1 attempts to describe the nature of the impact of a nonlinear mode of action for the carcinogenicity of arsenic on the expected dose–response.

In Fig. 1 a linear risk estimate for arsenic carcinogenicity such as that used for the proposed arsenic MCL is depicted by the dashed straight line. The heavy solid curve provides an example of a more plausible (but highly speculative) nonlinear dose–response for arsenic in an average individual. The strongly nonlinear nature of this curve reflects the sharp transition that would be expected to occur in a particular individual's cells, from concentrations of arsenic with little effect to those at which inhibition of a key cellular protein becomes evident. The factors listed at the right of Fig. 1 have been suggested to alter the sensitivity of an individual to the carcinogenic effects of arsenic in drinking water (NRC, 1999). For example, dietary intake of inorganic arsenic would add to the risk from drinking water exposure, while deficiencies in key nutrients, such as selenium and choline, could potentially increase the sensitivity of an individual to the effects of arsenic. Similarly, individual differences in the metabolism and clearance of arsenic could alter the relationship between drinking water intake and tissue arsenic concentrations. An example of a potentially important source of individual variation is the polymorphism for glutathione transferase Pi (GST-Pi), a key enzyme in the metabolism of arsenic. Other risk factors could include genetic predispositions, as well as alterations in key genes (e.g., the P53 tumor suppressor gene) due to exposures to other environmental carcinogens. The narrow solid curves suggest how variation in these risk factors across a population could shift the dose–response curve for specific individuals in either direction from the population mean.

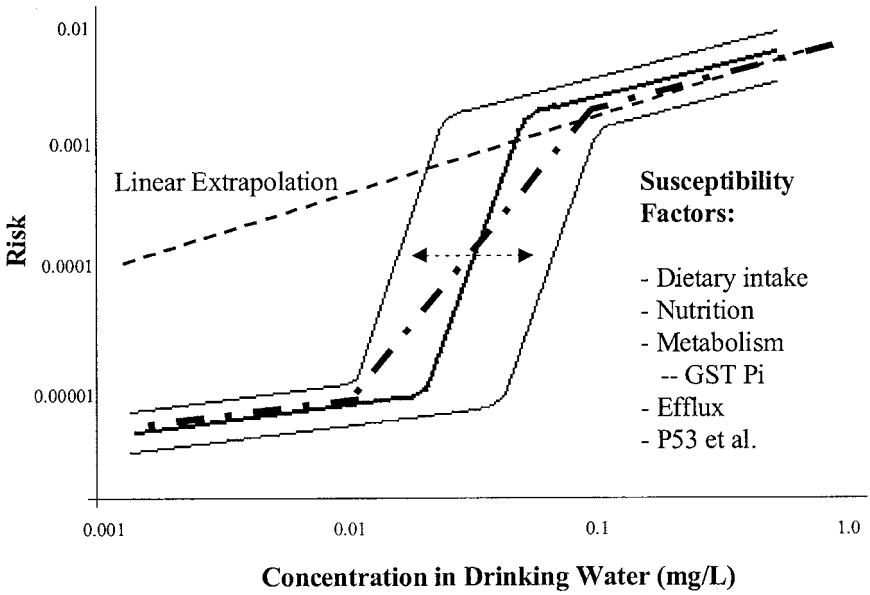

Fig. 1. Conceptual illustration of alternative dose–response curves for the carcinogenicity of arsenic in drinking water. GST Pi = glutathione transferase Pi isozyme.

The dose–response for the population would be a curve with a shallower slope, transitioning smoothly between the curves for the more and less sensitive individuals. This 'true' population risk is illustrated by the heavy dashed curve in Fig. 1. Thus the slope of the dose–response associated with the nonlinearity would depend on both the magnitude of the overall risk reduction in an individual due to the nonlinearity in the mode of action, and the breadth of the population transition resulting from inter-individual variability in sensitivity. The net result, as illustrated by comparing the curved and straight dashed lines, would be that lower arsenic exposures would result in a greater reduction in the population risk than would be predicted by the linear estimates.

Consideration of the implications of a sharply nonlinear dose–response on the risk estimates for arsenic carcinogenicity is particularly important in the case of risk estimates based on the Taiwanese population. The dose–response observed in the exposed Taiwanese villagers (fig. 1 in Morales et al., 2000) is a convex curve with a relatively shallow slope at low concentrations. If we assume that the 'true' dose–response for arsenic carcinogenicity is highly nonlinear, then there are two possibilities. First, it is possible that the nonlinearity occurs at a concentration below the range of exposures in the Taiwan study. In this case, the most appropriate dose–response estimates might be those calculated using a comparison group, yielding estimates of 1 in 100 risk at 0.01–0.02 mg/l (Morales et al., 2000). However, these extremely high risk estimates would seem to be at odds with the lack of evidence of carcinogenicity in U.S. populations exposed to relatively high concentrations of arsenic in drinking water (Lewis et al., 1999).

On the other hand, if the nonlinearity occurs in the range of the Taiwanese exposures, there must be some explanation for the lack of evidence of a clear nonlinearity in the observed dose–response in the exposed Taiwanese population. In fact, it has been suggested that the evaluation of the dose–response for cancers in the Taiwanese drinking water exposures is seriously compromised by limitations in the exposure assessments performed

in those studies (Brown et al., 1997). Specifically, it has been suggested that the use of the median well concentration measured in each village can inadvertently lead to mis-specification of personal exposures due to the extremely wide range of concentrations in the wells in many of the villages.

Because of the high salinity of shallow wells in this area, the studied population used deep artesian wells for drinking water; alternative water sources did not become generally available until 1970. The arsenic concentrations in the artesian wells were much greater than those in the shallow wells. Morales et al. (2000) used the median well concentration for each village, but mentioned that individual exposures could vary widely in a village. Brown and Chen (1995) had previously reviewed the data on well concentrations used as the basis for the exposure assessment for each village, and found that the range of concentrations was often several orders of magnitude, with lowest well concentrations around 0.01–0.05 mg/l and highest well concentrations around 0.6–1.75 mg/l. Given this variability, the median well could bear different relationships to the range of exposures from village to village. For example, in one village, where the range of well concentrations was 0.01–0.77 mg/l, the median well concentration was 0.03 mg/l, while in another village, with a range from 0.05 to 1.75 mg/l the median was 0.52 mg/l. Complicating the exposure estimates, many villages were under-sampled. That is, only a few wells in each village were actually tested. In most villages, only five or less wells were tested, and in twenty villages, only one well was tested.

A semi-quantitative evaluation of the dose–response for tumors in the Taiwanese population (Brown and Chen, 1995), taking into account information on the variation in well concentrations within villages, concluded that there was no evidence of excess risk of cancer mortality for exposure to drinking water arsenic at concentrations below 0.1 mg/l. Interestingly, a study of bladder and kidney cancer incidence in a different Taiwanese population, in which a more detailed personal exposure assessment was performed, also found evidence of a highly nonlinear dose–response, with effects of arsenic only being observed for exposures above 0.64 mg/l (Guo et al., 1994). These analyses suggest that it may actually be possible that the arsenic-induced tumors observed in the Taiwanese population were associated with personal exposures to well concentrations far above the current MCL. One way to evaluate the implications of this hypothesis would be to repeat the dose–response modeling for the exposed Taiwanese population under the assumption that each of the cancer outcomes was associated with the highest well concentration in the village, rather than the median. This would provide a 'lowest plausible' risk estimate that could be used to bound the uncertainty associated with mis-specification of exposure. A multivariate regression approach has also been proposed as more appropriate for dealing with uncertainty in exposure estimates in ecological studies where the dose–response is expected to be strongly nonlinear (Guo et al., 1998).

CONCLUSIONS

The current dilemma faced by the US EPA in conducting a cancer risk assessment for arsenic results from the lack of knowledge regarding the potential nonlinearity of the dose–response. Even if a specific nonlinear mode of action could be demonstrated, it is unlikely that the US EPA could justify departing from the linear default without some evidence that the nonlinearity would dramatically alter the cancer dose–response in the range of the drinking water concentrations of concern. Most of the currently proposed modes of action for the carcinogenicity of arsenic would be consistent with the expectation of a nonlinear dose–response; however, accurately determining the quantitative dose–response needed for a risk assessment will require further information regarding: (1) compelling evidence for a specific mode of action associating tissue exposure to one or more of the arsenic species with the eventual production of tumors; (2) the in vitro dose–

response for the critical cellular effect(s) of the relevant arsenic species; and (3) the tissue concentrations of the relevant arsenic species that result from human exposures.

Pharmacokinetic and biologically based dose–response modeling will play a crucial role in integrating these three kinds of information into a quantitative framework suitable for conducting a scientifically defensible risk assessment for arsenic. These same modeling approaches can also be used in the context of Monte Carlo analysis to evaluate the impact of inter-individual variability on the aggregate dose–response for the population. However, such an analysis will only be possible if the necessary studies have been performed to characterize the factors that influence the sensitivity of an individual to the carcinogenic effects of arsenic, including their quantitative impact as well as the extent of their variation within and across populations.

In summary, specific research activities that are needed to support the development of a more biologically plausible risk assessment for arsenic include the following.

(1) Mechanistic research to discriminate between the competing hypotheses for the mode of action of arsenic carcinogenicity and to provide quantitative information on the dose–response for the key events that determine the range of environmental exposures in which a nonlinearity could be expected to occur. This research is critical to permit the agency to depart from the linear default.

(2) Biologically based dose–response modeling of arsenic, including physiologically based pharmacokinetic modeling for tissue dosimetry and collection of data on the dose–response for the metabolism and protein interactions of the arsenic species. This capability is crucial to permit the estimation of a nonlinear cancer risk estimate as a quantitative alternative to the linear default.

(3) Research to identify and quantify the impact of the factors that modulate individual sensitivities to the carcinogenicity of arsenic. This information is necessary to determine an acceptable arsenic exposure that is protective for the general population, including sensitive individuals.

In the interim, the currently available linear risk estimates simply do not provide an adequate basis for choosing an MCL for arsenic from the range of options being considered. Unfortunately, the limited state of current knowledge concerning the nature of the dose–response for arsenic carcinogenicity makes it difficult to provide a more scientifically based evaluation of the appropriate concentration for an MCL. Considering the extensive research still required to support a scientifically sound risk assessment for arsenic, the most prudent approach at present would seem to be to reduce the MCL to the highest option considered, 0.02 mg/l. At the same time, a timetable could be presented for the completion of key additional research to support a more biologically plausible nonlinear risk assessment for arsenic in six years. If, at that time, the additional data did not provide the necessary assurance of an acceptable risk at the interim MCL, it could be further reduced. This step-wise strategy would serve to focus initial efforts on reducing the highest exposures, while providing an incentive for the collection of additional experimental data to make possible a more knowledge-based risk assessment for arsenic in the future.

ACKNOWLEDGEMENTS

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Arsenic Exposure and Health Effects IV
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The Discovery, Importance and Significance of Monomethylarsonous Acid (MMA^{III}) in Urine of Humans Exposed to Inorganic Arsenic

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ABSTRACT

Using a newer and more specific method of analysis (Le et al., 2000. Environ. Health Perspect., 108, 1015–1018), monomethylarsonous acid (MMA^{III}) has been detected for the first time in the urine of some humans exposed to inorganic arsenic in their drinking water. We studied subjects in Romania who had been exposed to 2.8, 28.5, 84.1 or 161 μg As/l in their drinking water. In the latter two groups, the MMA^{III} was 11 and 7% of the urinary arsenic, while the MMA^{V} was 14 and 13%, respectively. Of our 58 subjects, 17% had MMA^{III} in their urine. MMA^{III} was not found in the urines of any of the group with the lowest As exposure. The concentrations of MMA^{III} in male Golden Syrian hamster livers were determined 15 h after administration of a single intraperitoneal dose of [^{73}As]arsenate. Metabolites of inorganic arsenate present in hamster liver after 15 h were observed in the following concentrations (ng/g liver ± SEM); MMA^{III}, 38.5±2.9; DMA^{III}, 49.9±10.2; arsenite, 35.5±3.0; arsenate, 118.2±8.7; MMA^{V}, 31.4±2.8; and DMA^{V}, 83.5±6.7. MMA^{V} reductase activity, the rate-limiting enzyme of inorganic arsenic metabolism, was determined in tissues of male Golden Syrian hamsters. The specific activity of this enzyme in the brain > bladder > spleen > liver > lung > heart > skin > kidney > testis. This is the first identification of MMA^{III} in urine and MMA^{III} and DMA^{III} in liver and indicates that the significance of various arsenic species in mammalian tissue and urine needs to be re-examined and re-evaluated as to their role in the toxicity and carcinogenicity of inorganic arsenic.

Keywords: arsenic; metabolism; MMA^{III}; DMA^{III}; human; hamster

INTRODUCTION

Inorganic arsenic in the drinking water of humans is a major public health problem in many developing countries. Millions of people in Chile (Sancha et al., 1992), Mexico (Cebrian et al., 1983), Bangladesh (Science Scope, 1998), India (Guha Mazumder et al., 1988; Chatterjee et al., 1995), Taiwan (Chen et al., 1985), Romania (Surdu et al., 1997), and China (Chen et al., 1999) are at risk of hyperpigmentation, hyperkeratosis, skin cancer and cancers of the internal organs due to drinking water that contains excessive amounts of inorganic arsenic. There have been expensive and extensive epidemiology studies of this critical problem. Usually they have included measuring biomarkers such as arsenic species in the urine (Hopenhayn-Rich et al., 1996). But for some reason, a very crucial and toxic species of arsenic monomethylarsonous acid (MMA^{III}) (Fig. 1) has been overlooked and not measured.

At the 1998 meeting of this Society, we pointed out the need for careful study of: (a) the urinary concentrations of MMA^{III} in humans; (b) the properties of this neglected metabolite of inorganic arsenic; and (c) the need for re-examination and perhaps re-evaluation of the epidemiology of chronic inorganic arsenic toxicity in humans which has been previously based on findings that did not include MMA^{III}. The reasons for this oversight are unclear, but may be due to the investigators' lack of knowledge as to the potential importance of this much ignored biotransformant or they were not aware of suitable analytical methods for the analysis of MMA^{III} in human urine.

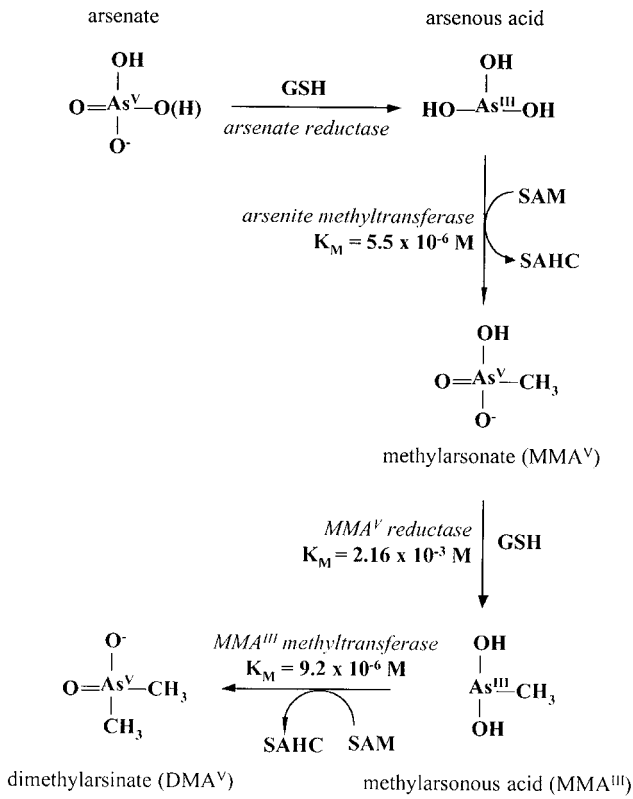

Fig. 1. The arsenic biotransformation pathway. SAM, *S*-adenosyl-L-methionine; SAHC, *S*-adenosyl-L-homocysteine.

TABLE 1

Demographic factors associated with subjects

	Locality			
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Population	10,000	1,600	2,757	2,056
Subjects, number	14	14	14	16
Women	7	7	7	8
Men	7	7	7	8
Drinking water				
Our analysis				
As^V ($\mu g/l \pm SD$)	2.8 [a]	23.5 ± 0.1 [b]	29.1 ± 0.7	87 ± 2
As^{III} ($\mu g/l \pm SD$)	nd	5.0 ± 0.3 [b]	55 ± 2	74 ± 1
Sum	2.8	28.5	84.1	161
Government data				
μg As/l	6	42	99	171
Depth of well, meters	100	370	150	200
Year established	1976	1904	1949	<1966

[a] Drinking water in this area is obtained from a central water facility dug in 1976 and 100 meters deep.
[b] This is water from the old well of this village. Of the 14 subjects in this group, all drank from this old well but four also drank water from a new well dug in 1986 which is 100 m deep and the government analysis was 58 μg As/l). By our analysis, the new well contained As^V, 46.8 ± 0.7 $\mu g/l$ and As^{III} could not be detected.

MMA IN HUMAN URINE

We can now report the occurrence of MMA^{III} in the urine of a number, but not all, of Romanian subjects drinking water containing different amounts of inorganic arsenic (Aposhian et al., 2000) These subjects had not previously received DMPS or any other metal-complexing agent as had our Chilean and Inner Mongolian subjects (Aposhian et al., 1997; Aposhian et al., 2000).

The subjects of our Romanian study were all in good health. The villages and towns where the subjects lived were in close proximity to the city of Arad in Arad County, Romania (Table 1). Water was collected by our team from each of the wells used by the subjects and analyzed by the same method used to analyze the urines (Le and Ma, 1998; Le et al., 2000). The well, from which each of our subjects obtained their drinking water, was easily identified. The results of our determination of the arsenic concentration of the water (Table 1) differed from the government results, probably because their collections and their assays had been performed in previous years as well as being determined by a less sensitive analytical procedure.

Although the number of subjects (14–16) in each group was small, there was, to some extent, a dose–response found as to arsenic in the drinking water and urinary MMA^{III} (Table 2). Groups A, B, C, and D correspond to the towns listed in Table 1.

When exposure was via drinking water containing 2.8 μg As/l, no urinary MMA^{III} was detected in the urine of the subjects (Table 2). At 28.5 μg As/l of drinking water, 7% of the group had MMA^{III}; at 84.1 μg As/l, 29%; and at 161 μg As/l, 31% had MMA^{III} in their urine. Group C and D with the higher As concentrations in their drinking water had more MMA^{III} in the urine (Table 2). No conclusions can be reached based on the results of Group A and B who drank water at the lower As concentration. Our interest was in the total MMA^{III} excreted and not the concentration. The concentration data are given, however, (Table 2) for those to whom they may be of interest.

TABLE 2

MMA^{III} in human urine

Group	Water (μg As/l)	Number in group	Subjects having MMA^{III} in urine	MMA^{III} in urine [a]	
				(μg/11 h ± SE)	(μg/l ± SE)
A	2.8	14	0 [b]	nd	nd
B	28.5	14	1 [c]	12.0	4.8
C	84.1	14	4 [d]	4.5 ± 1.5	5.7 ± 2.2
D	161	16	5 [e]	5.1 ± 1.6	6.9 ± 2.6

nd = not detectable.

[a] The values in the last two columns are the means only of those urines in which MMA^{III} was detected.

[b] No subject in this group had detectable amounts of MMA^{III} in the urine.

[c] One male.

[d] One male and three females.

[e] Four males and one female.

TABLE 3

Urinary arsenic species

Water (μg As/l)	Inorg As (%)	MMA^{V} (%)	MMA^{III} (%)	DMA (%)	Summation (%)
84.1	7	14	11	76	108
161	7	13	7	76	103
Literature	12–20	←14–18→		63–70	89–108

The summations are greater than 100% because they are summations of the means.

Many previous studies by others often have converted their urinary arsenic species data to percent of the total urinary arsenic (Vahter, 1998). In our case, if the MMA^{III} had not been determined, we assume that the MMA% would have been 25 and 20 (the sum of MMA^{V}% and MMA^{III}%, Table 3) for groups C and D, respectively (Table 3). Now that we have determined the individual MMA^{V} and MMA^{III} percentages, it can be stated that 44% and 35% of the total MMA was MMA^{III} in groups exposed to 84.1 μg As/l and 161 μg As/l, respectively.

It is of interest to note the studies that led to the discovery of the importance of MMA^{III}. In one of our previous studies, in which the chelating agent DMPS was given to native Chileans who had been chronically exposed to 600 μg inorganic arsenic per liter in their drinking water, a hint of an unknown peak was observed on an HPLC chromatogram during the analyses of their urines (Aposhian et al., 1997). Subsequently, after DMPS was given to arsenic exposed subjects in Inner Mongolia, MMA^{III} was identified in the urine of some but not all subjects exposed to inorganic arsenic (Aposhian et al., 2000). But for those subjects, however, the MMA^{III} was observed in the urine only after DMPS administration, raising the question as to whether MMA^{III} was an endogenously formed metabolite or whether DMPS chemically reduced the MMA^{V} to MMA^{III} in the urine. In vitro experiments indicated that the MMA^{III} was not formed by non-enzymatic chemical reduction via of DMPS (Aposhian et al., 2000). The development of a new, highly sensitive and reliable method for analysis of arsenic species in urine by Le et al. (2000) allowed many questions to be easily answerable.

It is worthy of note that only a limited amount, if any, of the studies of MMA^{III} could have been accomplished by any group without the contribution of the Cullen laborat-

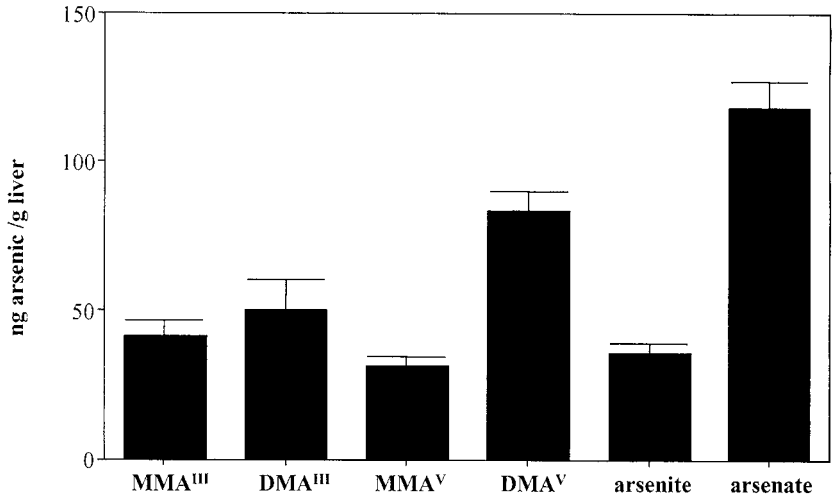

Fig. 2. Concentrations of arsenic species in hamster liver 15 h after a single i.p. dose of 145 μCi [^{73}As]arsenate, 2 mg As/kg body weight. Values represent the mean ± SEM of three hamsters. The following differences were significant using ANOVA with the Tukey–Kramer multiple comparisons post-test: $P < 0.001$ for DMA^{V} vs. MMA^{III}, arsenate vs. arsenite, arsenate vs. MMA^{V}, arsenate vs. DMA^{III}; $P < 0.01$ for DMA^{V} vs. arsenite, DMA^{V} vs. MMA^{V}; $P < 0.05$ for DMA^{V} vs. DMA^{III}, arsenate vs. DMA^{V}. All other differences were considered insignificant ($P > 0.05$).

ory and the Le laboratory. Professor Cullen has been very generous in making available chemically synthesized MMA^{III} and other arsenicals to many investigators. In addition, the inhibitory activity of MMA^{III} was first reported by him (Cullen et al., 1989), but unfortunately ignored or overlooked by those investigating arsenic toxicology in mammals. Professor Le has developed the HPLC–hydride generation–atomic fluorescence method for analyzing the arsenic species (Le and Ma, 1998; Le et al., 2000) and has generously undertaken the analysis of many of the urines of our studies. In addition, his method has pointed out clearly the limitations of the hydride generation–atomic absorption method that has been used so often in the past.

MMA^{III} AND DMA^{III} IN LIVER OF HAMSTERS

Fifteen hours after an injection of [^{73}As]arsenate into hamsters, trivalent arsenicals were extracted from the liver (for detailed procedure see Sampayo-Reyes et al., 2000). MMA^{III} was shown to be present (Fig. 2). The presence of trivalent methylated arsenicals in hamster liver was confirmed using anion exchange chromatography (Sampayo-Reyes et al., 2000). Of the hepatic retention, approximately 11% of [^{73}As] was present as MMA^{III}, 14% as DMA^{III}, 9% as MMA^{V}, 23% as DMA^{V}, 10% as arsenite and 33% as arsenate (Fig. 2). Arsenate and DMA^{V} were present in the greatest amount per gram tissue. This is the first demonstration of the previously ignored trivalent MMA^{III} and DMA^{III} in animal tissue.

ENZYMOLOGY OF INORGANIC ARSENIC BIOTRANSFORMATION

Our laboratory also has extensively studied the enzymes responsible for the biotransformation in mammalian systems of inorganic arsenic to dimethylarsinic acid (Fig. 1) (Aposhian, 1997; Zakharyan et al., 1995, 1996, 1999; Zakharyan and Aposhian, 1999a,b; Wildfang et al., 1998). Our approach at first has been as reductionists. We first purify away inhibitory

or stimulatory activities that cloud and conflict with the enzyme activity being studied. Then we can study the properties of the enzymatic reactions without ambiguity. A major result of this part of our investigations has been the finding that MMA^V reductase which catalyzes the reduction of MMA^V to form MMA^{III} is the rate-limiting enzyme of inorganic arsenic biotransformation (Fig. 1). We have previously reported that MMA^{III} is more toxic than inorganic arsenite (Petrick et al., 2000) in Chang human hepatocytes. Petrick's choice of a test system was important because, after all, understanding what happens in the human is the purpose of most basic science health research and it is why our laboratory studies inorganic arsenic metabolism in humans when possible. When human tissue is unavailable, rabbit and hamster are considered to be good models for what happens in the human (Marafante and Vahter, 1987; Maiorino and Aposhian, 1985). The rat is atypical for arsenic studies since a large amount of its DMA is bound to red cells which is not the case in humans. Studies of arsenic metabolism in rats have little, if any, value as a model for what happens in the human and consideration or reliance on studies with the rat can only confuse and delay an understanding of the complex metabolic pathway of inorganic arsenic.

We have partially purified a unique enzyme, MMA^V reductase, from rabbit and human liver by using DEAE cellulose, carboxymethylcellulose and red dye ligand chromatography (Zakharyan and Aposhian, 1999b). The enzyme is unique since it is the rate-limiting enzyme in the biotransformation of inorganic arsenite in rabbit liver (Zakharyan and Aposhian, 1999b). The K_m of MMA^V reductase has the largest value of all the enzymes of the inorganic arsenic biotransformation pathway. The enzyme has an absolute requirement for GSH. Other thiols such as DTT or L-cysteine were inactive alone. At a pH below the physiological, GSH carried out this reduction, but this GSH reduction in the absence of the enzyme had little if any value at pH 7.4. For MMA^V reductase, the MMA^V concentration must reach the millimolar range. Otherwise most of the MMA^V will be excreted in the urine or stored in the tissues. Thus, it would appear that the percent of each of the arsenic species found in the urine may be dependent on: (1) the concentration of MMA^V in the cell; (2) the rate of the MMA^V reductase; (3) the rate of MMA^{III} methyltransferase; and (4) the rate of urinary excretion of MMA^V (Fig. 1). At low concentrations in the cell, MMA^V can be expected to be excreted, or perhaps stored and/or bound to tissue. When arsenic ingestion increases, in vivo MMA^V concentration increases and reaches a critical *mM* level, then MMA^{III} begins to be made in larger amounts. Some MMA^{III} may accumulate in tissues, leading to MMA^{III} toxicity; some may be excreted; and some is converted to DMA^V. The control and regulation of this complex set of reactions needs more investigation. It is also possible that some MMA^{III} or DMA^V is demethylated. MMA and DMA can be demethylated by some bacteria and fungi (Cullen, personal communication). It is pertinent to re-emphasize that experiments using partially purified MMA^V reductase, disclosed that the concentration of MMA^V required for the reaction to reach half maximum velocity is in the millimolar concentration range, whereas that for the other enzyme reactions of the inorganic arsenite biotransformation pathway are in the micromolar range (Zakharyan and Aposhian, 1999b).

Another source of high levels of MMA^{III} in the urine may be due to polymorphic, less active MMA^{III} methyltransferase. Indisputable genetic or molecular biology evidence, however, has not been found for polymorphism in the biotransformation of arsenic. Such attempts (including our own) to attribute inexplicable results of arsenic experiments in humans to polymorphism are speculative. Polymorphism and its results have been carefully defined by geneticists and biochemists. We have found two major peaks of arsenic methyltransferase activity while purifying on DEAE cellulose and other ion-exchange columns. The two peaks of enzyme activity have similar substrates and other similar properties.

MMA^V reductase has also been purified from surgically removed human liver and its amino acid sequence determined (Zakharyan et al., 2001).

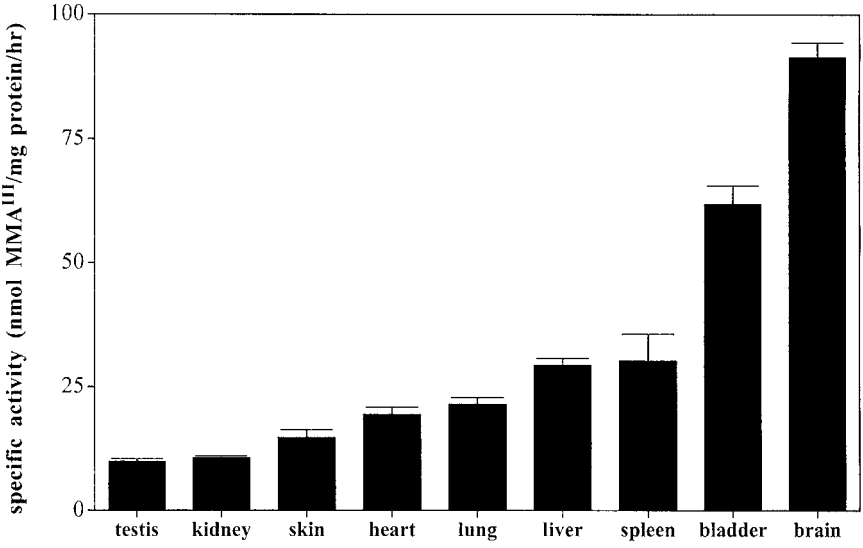

Fig. 3. MMA^{V} reductase activity in hamster tissues. Values represent the mean ± SEM of $n = 4$ animals.

MMA Reductase of Hamster Tissues

MMA^{V} reductase activity was observed in testis, kidney, skin, heart, lung, liver, spleen, bladder and brain of hamsters (Sampayo-Reyes et al., 2000). The results are based on the rate (limiting amounts) of MMA^{V} reductase, and not the limit (enzyme excess). Brain had the greatest enzyme activity and testis, the least (Fig. 3). The bladder, a tissue devoid of arsenic methyltransferase activity but a target for arsenic carcinogenicity, was very high in reductase activity.

DISCUSSION

Since MMA^{V} reductase is the rate-limiting enzyme of inorganic arsenic metabolism, its properties in individual humans may be the reason for the variability in response of individuals (NRC, 1999) to inorganic arsenic. Our studies further question the dogma created by earlier investigation that methylation of inorganic arsenic is a detoxication step. Our experiments clearly have shown that MMA^{V} reductase is found in mammalian liver and MMA^{III} in human urine and hamster tissue; that MMA^{III} is more inhibitory for Chang human hepatocytes than is arsenite (Petrick et al., 2000) and that it is more toxic than arsenite in the hamster as shown by whole animal LD50 studies (Petrick et al., 2001). The inhibition of GSH reductase by arsenicals has been reported (Styblo et al., 1997). In addition Huff et al. (2000) have recently proposed that DMA may be the ultimate carcinogen of the arsenic species. All these results plus the studies of Yamanaka et al. (1995) strongly argue against methylation of arsenic as a detoxication process.

The many enigmas of arsenic metabolism, mechanisms of carcinogenicity and dose–response relationships are beginning to be addressed and answered. This can be more easily done by interdisciplinary studies using the procedures and techniques of enzymology, toxicology, analytical chemistry and synthetic chemistry, molecular biology and epidemiology. In this way, perhaps some of the misery of economically poor children drinking water containing carcinogenic amounts of arsenic in developing countries can be decreased and even prevented.

The results of our studies in humans and our in vitro studies clearly indicate the importance of MMA^{III} which is formed endogenously and which is the most toxic arsenic species in the human.

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Arsenic Exposure and Health Effects IV
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The Impact of Selenium Status on the Metabolism and Disposition of Arsenic and its Implications for Epidemiologic Investigations

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ABSTRACT

Metabolic and toxicologic interactions between arsenic (As) and selenium (Se) are multifaceted and complex. These interactions are of practical significance because many populations are simultaneously exposed to inorganic As and varying levels of Se via both diet and drinking water. The goal of our studies has been to investigate whether dietary Se status alters As metabolism and disposition after exposure to arsenate [As(V)] or arsenite [As(III)]. Weanling female B6C3F$_1$ mice were maintained for 28 days on Torula yeast based diets deficient (0.02 ppm Se), sufficient (0.2 ppm Se) or excessive (2.0 ppm Se) in Se; mice then received by gavage either 0.5 or 5 mg (As)/kg as [^{73}As]sodium arsenate or 0.5 mg (As)/kg as [^{73}As]sodium arsenite. Se-deficient mice dosed with 5 mg (As)/kg As(V) exhibited slower whole body clearance from 8 h onward. Total (urine + feces) cumulative excretion of As-derived radioactivity was significantly lower in Se-deficient As(V)-exposed mice at both dose levels compared to Se-sufficient mice. Significantly less As-derived radioactivity was also excreted in the feces of Se-deficient mice exposed to 5 mg (As)/kg As(V) compared to Se-sufficient mice. There was also a trend towards lower cumulative excretion of dimethylarsinic acid in urine of Se-deficient compared to Se-sufficient mice which was significant for mice exposed to As(III). Both thioredoxin reductase activity and total hepatic Se were significantly increased in Se-deficient mice treated with As(III) compared to untreated Se-deficient mice. Overall, these studies indicate that Se deficiency is associated with altered As metabolism and disposition. Further studies to evaluate the potential toxicological consequences of As exposure in Se deficiency are warranted, as is consideration of population Se status in the design and interpretation of epidemiologic studies.

Keywords: arsenic; selenium; nutrition; metabolism

BACKGROUND

Selenium (Se) is an essential element that is incorporated in both glutathione peroxidase (GPx) and thioredoxin reductase (TR) as selenocysteine. Both enzymes play a central role in maintaining intracellular redox balance and protecting tissues from oxidative damage (Brigelius-Flohe, 1999; Mustacich and Powis, 2000). Diseases associated with both deficient and excess Se ingestion are well-documented in livestock and humans. This is due to uneven Se distribution in soil and the capacity of certain grasses and herbaceous plants to accumulate Se. Acute and chronic toxicity associated with excess dietary Se occurs in both animals and humans in the form of neurological and neuromuscular symptoms and skin lesions. Se deficiency is associated with nutritionally induced congenital muscular dystrophy (lambs, calves), liver necrosis (rats, mice), exudative diathesis (poultry), and heart failure due to skeletal and cardiac muscle necrosis (mice). Keshan disease, a form of cardiomyopathy endemic in certain areas of China, is a syndrome definitively associated with Se deficiency in humans (ATSDR, 1995; Goyer, 1996).

Arsenic (As) has been reported to be an essential element in some animal species on the basis of growth retardation in animals fed purified diets, however no essential biochemical function has ever been identified for As (U.S. EPA, 1988; NRC, 1999). Inorganic As occurs in drinking water in many areas of the world at levels sufficient to cause adverse health effects in humans. In epidemiologic studies, chronic ingestion of As-contaminated drinking water has been strongly associated with the development of peripheral vascular diseases, and cancers of the skin, lung, liver, kidney and urinary bladder in humans (NRC, 1999).

As and Se are both metalloids that are metabolized by a series of reductions and oxidative methylation steps to mono-, di- and tri-methylated species (ATSDR, 1995; NRC, 1999). Reduction is required prior to methylation for both As and Se (Lerman et al., 1983; Cullen et al., 1984a,b; ATSDR, 1995), but the relevant reactions occur by somewhat different mechanisms. Glutathione (GSH) provides reducing equivalents and forms both arsenoglutathione complexes and selenoglutathione complexes. Selenoglutathione complexes are subsequently reduced enzymatically in two steps to selenide (H_2Se) by glutathione reductase (ATSDR, 1995). However, GSH reductase does not catalyze the analogous reactions with arsenoglutathione complexes (Styblo and Thomas, 1995). Pentavalent arsenic species are reduced to trivalent arsenic species either chemically using GSH or enzymatically by arsenate reductases(Scott et al., 1993; Delnomdedieu et al., 1994; Radabaugh and Aposhian, 2000). The relative contribution of each mechanism to arsenic reduction in mammalian species in vivo is not known with certainty. As and Se both undergo enzymatic methylation using *S*-adenosylmethionine (SAM) as the cofactor, but different enzymes appear to be involved for each metalloid (Styblo et al., 1996).

There are a number of possible points and mechanisms for metabolic interaction between As and Se. These include competition for the methyl donor (SAM), competition for GSH, and inhibition of GSH reductase by a number of trivalent arsenicals and arsenothiols (Styblo and Thomas, 1995; Styblo et al., 1997). Interaction at the level of Se methylation is plausible because arsenite inhibits selenium methylation both in vivo and in vitro (Ganther and Baumann, 1962; Tandon et al., 1986; Hoffman and McConnell, 1987). Se(IV) is also a potent inhibitor of As methylation in vitro (Styblo et al., 1996).

Toxicologic and metabolic interactions between As and Se have been widely reported in the literature. Arsenic is generally protective against the toxicity of most forms of Se (e.g. selenate, selenite, selenocysteine, selenomethionine) in laboratory and farm animals (Levander, 1977). Accumulated evidence suggests that this protective effect is mediated by dispositional antagonism (Ganther and Baumann, 1962; Levander and Baumann, 1966a; Ganther and Hsieh, 1974). Studies in bile duct cannulated rats and guinea pigs have demonstrated that As causes increased biliary excretion of Se (Levander and Baumann, 1966b). Levander (1977) suggested that As enhances the biliary excretion of Se via forma-

tion of a 'detoxification conjugate', that is excreted in the bile. It has also been suggested that GSH could be involved in this process since biliary excretion of As as either arsenate [As(V)] or arsenite [As(III)] is dependent on hepatobiliary transport of GSH (Gyurasics et al., 1991). A recent report by Gailer et al. (2000) supports this hypothesis because they identified a seleno-bis(*S*-glutathionyl)arsonium cation in the bile of rabbits injected with selenite followed by As(III). Berry and Galle (1994) have also demonstrated that As and Se combine directly in vivo and are concentrated and precipitated in rat renal lysosomes as insoluble As_2Se.

A strong synergistic toxicity of As with certain methylated selenium compounds has also been reported in rats. Obermeyer et al. (1971) reported that 4 mg/kg As(III) increased the lethality of trimethylselenonium chloride [$(CH_3)_3SeCl$] ~20-fold in rats when both were administered simultaneously by i.p. injection. Similarly, Kraus and Ganther (1989) reported that simultaneous administration of 4 mg/kg as As(III) increased mortality and toxicity of a number of methylated Se compounds including Se-methylselenocysteine, methylseleninic acid, selenobetaine, selenobetaine methyl ester and dimethylselenoxide in rats. Rats simultaneously injected with periodate-oxidized adenosine (PAD), a generalized inhibitor of transmethylation, and methylated Se compounds show a comparable enhancement of toxicity (Kraus and Ganther, 1989). Based on this finding, the authors suggested that arsenite may block detoxification of methylselenides by inhibiting further methylation to the trimethylselenonium ion.

A few studies of As and Se interactions have focused on the ability of Se to prevent or ameliorate arsenical-induced toxicity in experimental animals supplemented with extra Se (Flora et al., 1999; Biswas et al., 1999). In general, these studies have demonstrated that Se can ameliorate the toxicity of As compounds, if Se is given before or concurrently with the administration of As. For example, Biswas et al. (1999) reported that oral administration of sodium selenite (5.6 mg/kg) 1 h before administration of sodium arsenite (2.5 mg/kg) significantly decreased the frequency of chromosomal aberrations in bone marrow cells of Swiss albino mice compared to mice given the same dose of arsenite alone. No such reduction was observed when arsenite was given before selenite and a smaller effect was noted when both metalloids were given simultaneously.

Interactions between As and Se are clearly complex and relevant to public health because there are numerous areas throughout the world in which populations are simultaneously exposed to relatively high levels of inorganic As, mainly as As(V), in drinking water, together with varying levels of Se in both the diet and drinking water. In this paper, results from studies conducted in our laboratory on the impact of Se status on As metabolism and disposition in mice are reviewed and their implications for epidemiologic studies are discussed.

METHODOLOGY

A study design (Fig. 1) was employed in which the selenium status of female B6C3F1 mice was altered by feeding one of three Torula yeast based diets (Se-deficient, Se-sufficient or Se-excess). The effect of this alteration on As metabolism and disposition was evaluated following an acute oral dose of As (Kenyon et al., 1997, 1999). Whole body clearance of [^{73}As]arsenic derived radioactivity was determined by whole body counting immediately after As dosing and at intervals until sacrifice. Ion-exchange chromatography was used to analyze cumulative 24-h urine samples for monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). Reverse phase ion-pairing HPLC was used to analyze urine for As(III) and As(V) (Hughes and Thompson, 1996). Total tissue As and total Se were determined by hydride generation atomic absorption spectrophotometry (HGAAS). The liver samples were completely wet digested in open vessels with sequential addition of nitric, sulfuric, and perchloric acids (Cox, 1980). This procedure converts all arsenicals and selenicals to

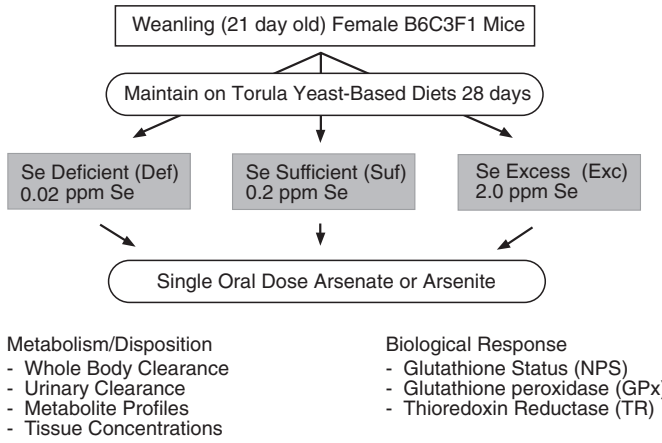

Fig. 1. Experimental design in the Kenyon et al. (1997, 1999) studies.

inorganic As and Se for HGAAS determination, respectively. All analyses were made using As and Se electrodeless discharge lamps at 197.3 and 196.0 nm, respectively, in a heated quartz cell.

Serum Se levels were used to verify Se status and were determined by Zeeman atomic absorption spectrophotometry as described previously (Levander et al., 1989). Serum Se was determined in both mice fed the diets and not exposed to As (control), and mice fed the diets and dosed with unlabeled As(V) and As(III). Nonprotein sulfhydryl (NPS) content, GPx activity and TR activity were determined in livers of these same animals to assess tissue oxidative status. NPS content was determined spectrophotometrically using Ellman's reagent (Sedlak and Lindsay, 1968). Hepatic GPx activity was measured as described by Levander et al. (1983). TR activity was determined by a modification of the method of Tamura and Stadtman (1996) using gold thioglucose to correct for non-thioredoxin reductase dependent 5,5′-dithiobis(2-nitrobenzoic acid) reduction as described by Hill et al. (1997). One milliunit of TR activity is defined as 1 nmol 2-nitro-5-thiobenzoic acid formed per minute.

RESULTS AND DISCUSSION

In an initial study, we (Kenyon et al., 1997) compared the 48-h time course of urinary arsenic metabolite excretion in selenium deficient, sufficient and excess mice given a single oral dose of 5 mg (As)/kg as sodium arsenate. One finding was that mice on the selenium excess diet excreted a significantly higher percentage of urinary As as inorganic As ($41.8 \pm 3.7\%$ vs. $32.2 \pm 1.5\%$) with a significantly decreased ratio of organic to inorganic As (1.41 ± 0.23 vs. 2.12 ± 0.14) compared to Se-sufficient mice. This suggests that As methylation was decreased or slowed in Se-excess mice compared to Se-sufficient mice in this study. This might be explained on the basis of either Se competing more effectively for SAM or depletion of SAM pools. However, since the mice had been maintained on the high Se diets for over a month, depletion of SAM pools is a more likely explanation. Another finding of interest in this study (Kenyon et al., 1997) was that in mice on the Se-deficient diet, arsenate and arsenite elimination peaked later and dimethylarsinic acid excretion was more protracted compared to mice on the Se-sufficient diet.

In a subsequent study using this same basic design (Fig. 1), we evaluated the effect of Se status on whole body clearance and cumulative 24 h excretion of As metabolites in urine following a single oral dose of 0.5 mg (As)/kg as either sodium arsenate or

TABLE 1

Cumulative urinary excretion of As metabolites in Se-deficient (Def), Se-sufficient (Suf) and Se-excess (Exc) mice 24 h after oral dosing with As

Arsenic (mg/kg)	Se status	Metabolite (% of dose)			
		Arsenate	Arsenite	MMA	DMA
As(III) 0.5	Def	<LD	1.14 ± 0.31	0.45 ± 0.05	67.8 ± 4.38*
	Suf	<LD	1.30 ± 0.30	0.52 ± 0.20	77.6 ± 4.93
	Exc	<LD	1.46 ± 0.51	0.58 ± 0.14	72.7 ± 2.23
As(V) 0.5	Def	12.3 ± 1.91	0.85 ± 0.18*	0.47 ± 0.18	58.6 ± 5.22
	Suf	13.8 ± 2.61	<LD	0.45 ± 0.15	62.2 ± 6.34
	Exc	15.6 ± 2.97	<LD	0.50 ± 0.14	64.7 ± 2.23
As(V) 5.0	Def	18.8 ± 4.10	6.28 ± 1.28	0.81 ± 0.09	43.1 ± 2.16
	Suf	22.4 ± 2.76	5.39 ± 1.00	0.88 ± 0.35	45.4 ± 6.24
	Exc	17.7 ± 8.11	4.68 ± 1.81	0.82 ± 0.05	42.6 ± 4.25

Figures are mean ± SD for $n = 5$ mice. <LD indicates less than limit of quantification of ~0.05% of dose.
* Significant difference ($P < 0.05$) for deficient or excess compared to sufficient.

sodium arsenite and 5 mg (As)/kg as sodium arsenate (Kenyon et al., 1999). Se status did not affect whole body retention of As in mice dosed with either As(V) or As(III) at 0.5 mg (As)/kg. However, in Se-deficient mice dosed with 5 mg (As)/kg as As(V), whole body clearance was slower from 8 h onward. Total (urine + feces) cumulative excretion of As-derived radioactivity was significantly lower in Se-deficient arsenate-exposed mice at both dose levels. Significantly less As-derived radioactivity was also excreted in the feces of Se-deficient mice exposed to 5 mg (As)/kg as sodium arsenate compared to Se-sufficient mice. There was also a trend toward lower cumulative excretion of dimethylarsinic acid in urine of Se-deficient compared to sufficient mice which was significant for mice exposed to As(III) (Table 1).

There are a number of possible explanations for reduced or delayed As elimination under conditions of Se deficiency observed in both studies. Given that both Se(IV) and As(III) interact extensively with −SH groups in tissues, it is possible that As elimination is delayed in Se-deficient mice because there could be more target −SH groups for As to react with when Se intake is low. Since As(V) is also reduced to As(III) prior to methylation (Marafante et al., 1985; Wood et al., 1978; Cullen et al., 1984a,b), these aggregate observations could also be explained by slower reduction of As(V) to As(III) in Se-deficient mice. Slower As(V) reduction might be expected if the supply of GSH were limiting. This is because GSH reduces As(V) to As(III) and is also involved in the analogous reduction reactions with pentavalent MMA and DMA (Scott et al., 1993; Delnomdedieu et al., 1994).

In our 1999 study, the impact of As administration on cellular redox status in the presence of altered Se status was also evaluated (Kenyon et al., 1999). Hepatic GPx activity and GSH were unaffected by As administration irrespective of Se status. However, As administration resulted in what might be termed a 'partial restoration effect' of hepatic TR activity in Se-deficient mice. Specifically, TR activity was increased in Se-deficient mice dosed with As compared to control mice at 6 h post-dosing and this was statistically significant for mice dosed with As(III). At 18 h post-dosing, this effect was no longer apparent. No similar effect was noted in Se-sufficient or Se-excess mice (Table 2). Since Se deficiency results in decreased hepatic TR activity in rats and Se repletion will increase TR activity by ~3-fold within 12 h (Hill et al., 1997), an increase in TR activity in the short term after As administration in Se-deficient mice might be hypothesized to result from displacement of sequestered Se by As.

TABLE 2

Hepatic TR (mU/mg protein) activity* in control and arsenic-treated mice 6 h after dosing**

Selenium diet group	Arsenic form and dose level (mg(As)/kg)			
	Control	As(III) 0.5	As(V) 0.5	As(V) 5.0
Deficient	0.61 ± 0.20	1.42 ± 0.27**	0.97 ± 0.58	1.13 ± 0.65
Sufficient	4.16 ± 1.34	4.30 ± 0.53	5.07 ± 0.81	4.36 ± 1.34
Excess	3.73 ± 1.04	4.67 ± 1.01	4.67 ± 0.29	4.26 ± 0.85

* One milliunit of TR activity is defined as 1 nmol 2-nitro-5-thiobenzoic acid formed per minute.
** Significantly different ($P < 0.05$) for comparison of controls to As-treated mice within Se diet group. No significant effect of As treatment noted at 18 h post-dosing.

TABLE 3

Total As and total selenium in livers of control and arsenite-treated mice 6 h after oral dosing

Selenium diet group	Total arsenic (μg/g)		Total selenium (μg/g)	
	Control	As(III) 0.5 mg/kg	Control	As(III) 0.5 mg/kg
Deficient	0.009 ± 0.0013	0.203 ± 0.035	0.37 ± 0.030	0.62 ± 0.147*
Sufficient	0.009 ± 0.0023	0.252 ± 0.037	1.77 ± 0.192	1.77 ± 0.160
Excess	0.008 ± 0.0013	0.214 ± 0.050	2.09 ± 0.247	2.19 ± 0.319

* Significantly different ($P < 0.05$) for comparison of controls to As-treated mice within Se diet groups. Figures are mean ± SD for $n = 5$ mice.

The possibility that As administration mobilizes hepatic Se was investigated by determining both total As and total Se in control and As(III) treated mice from all the Se diet groups. Consistent with the hypothesis that As(III) administration mobilizes Se, total hepatic Se was significantly elevated in arsenite-treated Se-deficient mice compared to controls by nearly twofold, but not in Se-sufficient or excess mice (Table 3). The mechanism and form of elevated hepatic Se in arsenite-treated Se-deficient mice compared to control mice could not be determined in this study since the limited amount of liver sample allowed for analysis only of total Se. The possibility remains that the increased TR activity in response to As(III) administration may reflect induction by As(III) of increased production of reactive oxygen species and the fact that TR is a central enzyme in the cellular response to oxidative stress (Buchanan et al., 1994).

CONCLUSIONS AND RESEARCH NEEDS

The impact of dietary Se status on As metabolism is of interest from both the standpoint of exposure potential and toxicity. Numerous studies have investigated As and Se interactions at the level of both effects on metabolism and toxicity in laboratory animals, but generally focusing on the effect of arsenic on Se-induced toxicities. Studies in humans have primarily focused on the correlation of As and Se levels in tissues and excreta in both apparently healthy populations and patients suffering from As-induced peripheral vascular disease (Lin and Yang, 1988; Wang, 1996; Del Razo et al., 2000). Relatively few investigations have been conducted in which dietary Se status has been examined as a potential modifier of As metabolism and toxicity in laboratory animals or humans.

Se, in the form of selenocysteine, is a critical component of several enzymes that are

known to be important in the maintenance of intracellular redox balance, including TR and GPx (Hill, 1994; Holben and Smith, 1999). This is relevant to As in that oxidative stress is one of the hypothesized mechanisms by which As, and in particular As(III), is believed to induce cytotoxic, genotoxic and carcinogenic effects (Chan and Huff, 1997). The knowledge that Se deficiency can exacerbate the toxicity of certain chemicals (Pence, 1991; Kays et al., 1992; Awad et al., 1994; Watanabe et al., 1999) and increase the pathogenicity of certain viruses (Beck, 1999) has enhanced interest in the potential of Se supplementation as a therapeutic or chemopreventive measure in various diseases in addition to cancer. Biswas et al. (1999) have recently suggested that dietary Se supplementation might be helpful in protecting against the toxicities associated with long-term exposure to arsenic in drinking water. However, many key questions remain unanswered with respect to the potential role of Se and Se status in modulating the adverse effects of arsenic exposure.

Studies in human populations are needed to determine the correlation of As (inorganic and methylated metabolites) and Se in blood and body fluids and how this may be altered depending on arsenic exposure and in the presence of arsenic-induced disease. Evaluation of Se status by functional biochemical measures such as GPx activity in human populations exposed to varying levels of arsenic in drinking water is also important. The need for such studies arises from the fact that how arsenic exposure and Se status are related and their interactions are poorly understood in human populations.

Two related but distinctly different questions that need to be answered are: (1) does high level As exposure and the presence of As-induced disease alter Se disposition and the function of selenoenzymes such that Se supplementation would be beneficial as a chemotherapeutic or chemopreventive measure; and (2) does Se deficiency itself constitute a risk factor for the development of As-induced disease in arsenic exposed populations. A combined strategy of mechanistic research and human clinical and epidemiologic studies will be necessary to address these questions.

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Arsenic Exposure and Health Effects IV
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Trivalent Methylated Arsenicals: Toxic Products of the Metabolism of Inorganic Arsenic

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ABSTRACT

Biomethylation is the major metabolic pathway for inorganic arsenic (iAs) in humans. In this pathway, iAs is metabolized to methylated arsenicals, including methylarsenic (MAs) and dimethylarsenic (DMAs) species. Because of the redox nature of the methylation reactions, both pentavalent and trivalent methylated arsenicals (iAs^V, iAs^{III}, MAs^V, MAs^{III}, $DMAs^V$, $DMAs^{III}$) are intermediates or final products in this pathway. Trivalent iAs^{III} and MAs^{III} are the preferred substrates for arsenic methyltransferase. Thus, the reduction of iAs^V to iAs^{III} and of MAs^V to MAs^{III} is a prerequisite for completion of the methylation process. Pentavalent methylated metabolites (MAs^V and $DMAs^V$) are less acutely toxic than either iAs^V or iAs^{III}. Therefore, methylation has commonly been assumed to be a mechanism for detoxification of iAs. However, recent evidence suggests that the trivalent methylated metabolites, MAs^{III} and $DMAs^{III}$, significantly contribute to the adverse effects which are associated with exposure to iAs. For example, MAs^{III} and $DMAs^{III}$ are more cytotoxic to human and animal cells in the cell lines tested and are by several orders of magnitude more potent enzyme inhibitors than is iAs^{III}. Recent advances in analytical techniques have provided evidence that trivalent methylated arsenicals are products of the biomethylation of iAs. Using these techniques, MAs^{III} and $DMAs^{III}$ were found in urine of human individuals exposed to iAs in drinking water, in tissues of hamsters exposed to iAs, and in human hepatic cells exposed to iAs in culture. Taken together, these data suggest that MAs^{III} and $DMAs^{III}$ are toxic products of the metabolism of iAs in humans. The analysis of urinary MAs^{III} and $DMAs^{III}$ may become a useful tool for the evaluation of the health risks associated with this exposure.

Keywords: arsenic; metabolism; methylated metabolite; toxicity; human

INTRODUCTION

Like most metals and metalloids, the toxicity of arsenic in biological systems depends strongly on its chemical form. Although inorganic arsenicals (iAs) are the most common forms of arsenic in the environment, organic species, especially methylated arsenicals, have been found in tissues and excreta of organisms exposed to iAs. Biomethylation of arsenic, the enzymatic conversion of iAs to mono-, di- and trimethylated metabolites, appears to be the major metabolic pathway in many mammalian species, including humans (for reviews see Vahter, 1994; Styblo et al., 1995a; Aposhian, 1997). Hence, the biological effects of arsenic in biomethylating species are likely to be determined by the chemical nature and biological activity of the intermediates and final products of the methylation reactions. The metabolic scheme for iAs proposed by Cullen et al. (1984) consists of two types of reactions, including reduction of pentavalent arsenicals to trivalency and subsequent oxidative methylation to yield methylated pentavalent species. Thus, both pentavalent and trivalent iAs (iAs^V and iAs^{III}), methylarsenic (MAs^V and MAs^{III}), dimethylarsenic ($DMAs^V$ and $DMAs^{III}$) and trimethylarsenic ($TMAs^V$ and $TMAs^{III}$) species are intermediary or final products of the pathway:

$$iAs^V \rightarrow iAs^{III} \rightarrow MAs^V \rightarrow MAs^{III} \rightarrow DMAs^V \rightarrow DMAs^{III} \rightarrow TMAs^V \rightarrow TMAs^{III}$$

In humans exposed to iAs from the environment, DMAs is likely the final product of the methylation pathway (Hopenhayn-Rich et al., 1993, 1996). For years iAs, in particular iAs^{III}, has been considered the most toxic among arsenicals found in methylating species. Because pentavalent products of the methylation reactions, MAs^V and $DMAs^V$, are significantly less acutely toxic than iAs (Yamauchi and Fowler, 1994), this pathway has been considered a major mechanism for detoxification of iAs. However, little was known about toxicity of trivalent methylated metabolites, MAs^{III} and $DMAs^{III}$. In 1989, Cullen and associates showed that MAs^{III} is highly toxic for cultured fungus (Cullen et al., 1989). In recent years, additional data on toxicity and other adverse effects of trivalent methylated arsenicals have been reported. In addition, both MAs^{III} and $DMAs^{III}$ were found in urine of individuals exposed to iAs in drinking water (Aposhian et al., 2000a; Del Razo et al., 2001), indicating that these arsenicals are indeed products of the metabolism of iAs in humans. These new findings strongly contradict the theory of biomethylation as the major mechanism for detoxification of iAs, suggesting that this pathway yields toxic intermediary products that can be in part responsible for adverse effects associated with the exposure to iAs.

This paper summarizes results of our work since 1995 on: (1) the characterization of the effects of MAs^{III} and $DMAs^{III}$ on activities of NADPH-dependent oxidoreductases; (2) examination of the cytotoxicity of MAs^{III} and $DMAs^{III}$ in animal and human cells; (3) examination of the metabolism and protein binding of MAs^{III} and $DMAs^{III}$ in tissue extracts and cultured cells; and (4) analysis of these arsenicals in biological samples. Our work complements studies in other laboratories. Taken together, the results of this research provide important clues for the reevaluation of the role of the methylation pathway in the toxicity and carcinogenicity of arsenic.

INHIBITION OF NADPH-DEPENDENT OXIDOREDUCTASES BY MAs^{III} AND $DMAs^{III}$

It has been previously shown that iAs species that contain trivalent arsenic (As^{III}) are relatively potent inhibitors of various enzymes, e.g., pyruvate dehydrogenase (PDH) (Peters, 1955) or glutathione reductase (GR) (Mize and Langdon, 1962). The mechanism of inhibition involves interactions between As^{III} and critical thiol groups of enzymes or of enzyme cofactor(s). For example, interactions of As^{III} with thiols of vicinal cysteinyl residues in the active site of GR are responsible for the inhibition of this enzyme by trivalent arsenicals (Knowles, 1985). On the other hand, inhibition of the PDH multienzyme complex

TABLE 1

GR Activity and total GSH in primary rat hepatocytes exposed to iAs^{III} or MAs^{III} for 30 min (mean ± SD, $n = 3$)

Concentration of arsenicals in culture	GR activity (U/mg protein)*	Total GSH** (nmol/10^6 cells)
Control	96.3 ± 6.79	27.6 ± 1.90
4 μM iAs^{III}	89.5 ± 5.82	28.4 ± 0.88
10 μM iAs^{III}	84.9 ± 0.46	23.8 ± 1.17***
50 μM iAs^{III}	71.4 ± 1.03***	22.1 ± 0.61***
1 μM MAs^{III}	92.5 ± 4.18	24.7 ± 2.77
5 μM MAs^{III}	73.7 ± 2.70***	20.6 ± 2.55***
10 μM MAs^{III}	72.6 ± 1.35***	19.7 ± 2.68***

The 30-min exposures used in this study did not significantly change cell viability with exception of the exposure to 10 μM MAs^{III}, which decreased cell viability by about 20%.

* Unit (U) is defined as nmol NADPH oxidized per minute.

** Total GSH represents a sum of the reduced and oxidized forms.

*** Values were found significantly different ($P < 0.05$) from control values by ANOVA and the Dunnett multiple comparisons post-test.

involves binding of As^{III} to thiol groups in the molecule of lipoic acid, a PDH cofactor (Adamson and Stevenson, 1981). Considering the high affinity of As^{III} towards thiols, organic trivalent arsenicals, including trivalent methylated species, are also likely to interfere with the activities of many thiol-dependent enzymes. We have previously examined the effects of iAs^{III}, iAs^{V} and tri- and pentavalent methylated arsenicals on activities of two major NADPH-dependent oxidoreductases, GR and thioredoxin reductase (TR). We found the trivalent methylated arsenicals, MAs^{III} and $DMAs^{III}$, to be more potent inhibitors of purified yeast GR than was iAs^{III} (Styblo et al., 1997). Among arsenicals examined, MAs^{III} complexed with glutathione (GSH) was the most potent inhibitor with $K_i \sim 9$ μM. The MAs^{III}-diglutathione complex ($MAs^{III}(GS)_2$) was two orders of magnitude more potent GR inhibitor than was iAs^{III}. MAs^{III} was also an extremely potent inhibitor ($K_i \sim 100$ nM) of TR purified from mouse liver (Lin et al., 1999), indicating that NADPH-dependent oxidoreductases may be specific targets for this trivalent arsenical. It is likely that MAs^{III} interacts directly with thiols of cysteinyl residues that are present in the active sites of both GR (Karplus et al., 1989) and TR (Holmgren, 1985). In addition to cysteinyl residues, TR contains a catalytically active selenocysteine (Zhong et al., 1998). Although selenocysteine is not a part of the active center of the enzyme, it is essential for its optimal activity. This selenocysteine residue is likely an additional target for MAs^{III} in the molecule of TR. The ability of As^{III} to directly react with selenium has previously been reported in cultured cells (Berry and Galle, 1994).

Glutathione reductase (GR) plays a critical role in the metabolism of GSH, maintaining a balance of the reduced and oxidized forms of this major cellular antioxidant. We have found the inhibition of GR in cultured cells exposed to MAs^{III} to be associated with a significant decrease in the intracellular GSH levels (Table 1). MAs^{III} was more potent in decreasing GSH levels in cells than was iAs^{III}. Similarly, TR catalyzes reduction of a number of dithiol-containing proteins, in particular thioredoxin (Trx) (Holmgren, 1985). Trx, a small 12-kDa protein, is an important part of the cellular redox mechanism that regulates responses to oxidative stress, including expression of oxidative stress-sensitive transcription factors (Ueno et al., 1999). For example, Trx has been shown to enhance binding of nuclear factor κB (NF-κB) to DNA (Hirota et al., 1999). Inhibition of the TR-catalyzed regeneration of Trx-disulfide by MAs^{III} would likely result in a suppression of the NF-κB binding activity with subsequent modification of the NF-κB-dependent gene expression.

In addition, TR activity is required for optimal functioning of the p53 tumor suppressor gene (Pearson and Merrill, 1998). In the absence of TR activity, p53 cannot function as a transcription factor. Thus, inhibition of GR and TR by MAs^{III}, an intermediary metabolite of iAs, may result in significant changes in the cellular redox status and in the modification of nuclear mechanisms involved in the regulation of cell growth, proliferation and death. The relationship between iAs metabolism and expression and DNA-binding of the oxidative stress-sensitive transcription factors, including NF-κB and p53, is currently under investigation in this laboratory.

CYTOTOXICITY OF MAs^{III} AND $DMAs^{III}$

In 1989, Cullen and associates reported that the trivalent methylated arsenicals, methylarsine oxide and methylarsine sulfide, were three to four orders of magnitude more toxic for *Candida humicola* than was iAs^{III}. The authors suggested that methylation of iAs can be a detoxification process only when toxic intermediary metabolites were not allowed to accumulate in cells. Cullen's group was first to raise the question of the role of methylation in the generation of toxic arsenic species. Ten years later, our laboratory presented the first data on acute toxicities of trivalent methylated arsenicals in mammalian cells (Styblo et al., 1999). We have examined the toxicities of iAs^{III}, iAs^{V} and tri- and pentavalent methylated arsenicals in cells derived from animal and human tissues (liver) that are known to methylate iAs and tissues that are targets for carcinogenic effects of iAs (skin, lung, and urinary bladder). Among arsenicals examined, MAs^{III} species (methylarsine oxide and methylarsine diiodide) were most cytotoxic with LC_{50} values ranging from 0.8 μM in human bladder epithelial cells to 2.8 μM in rat primary hepatocytes and 5.5 μM in human primary hepatocytes (Fig. 1). For most cell types, $DMAs^{III}$ species (dimethylarsine–GSH complex or dimethylarsine iodide) were more toxic than iAs^{III}. In human bronchial epithelial cells, iAs^{III}, MAs^{III} and $DMAs^{III}$ were almost equally toxic (Styblo et al., 2000). Pentavalent arsenicals, iAs^{V}, MAs^{V}, $DMAs^{V}$, were much less toxic for any cell type than were their trivalent counterparts (data not shown). Notably, there was no apparent correlation between the cytotoxicity of trivalent arsenicals and capacity of cell types to methylate arsenic. Cells that effectively methylated iAs^{III} to MAs and DMAs (e.g., primary rat hepatocytes) were about as sensitive to the toxic effects of iAs^{III} as were cells with low methylation capacities (e.g., human epidermal keratinocytes). Hence, methylation did not protect cultured cells against acute toxicity of iAs^{III}. Our findings were extended by Petrick

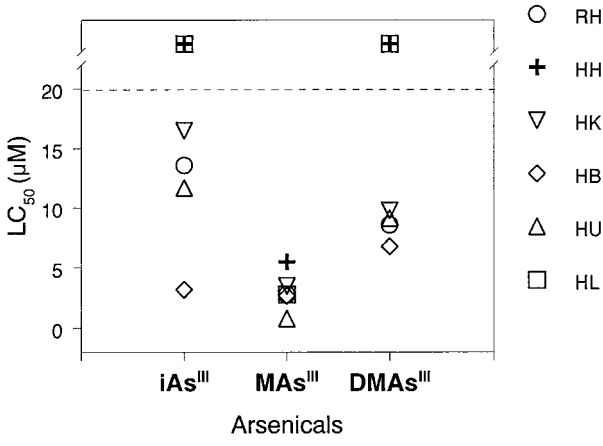
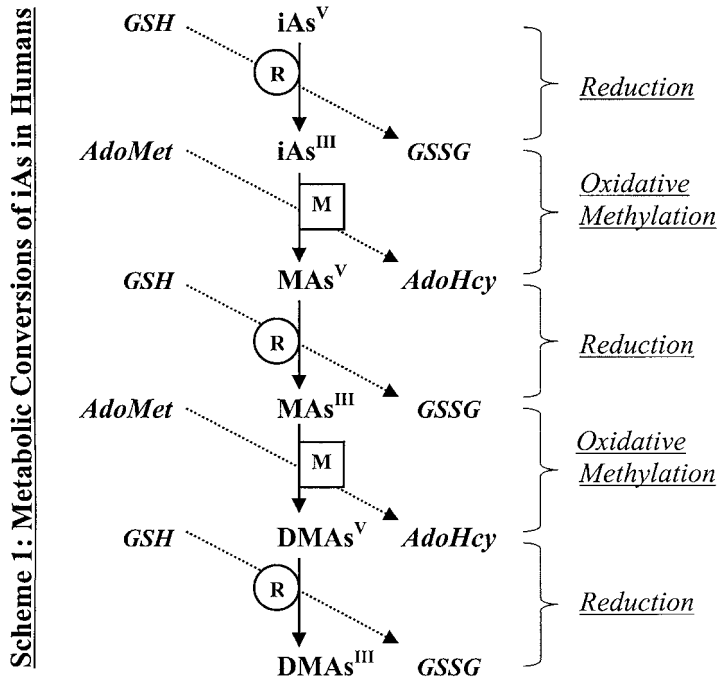


Fig. 1. Cytotoxicity of trivalent arsenicals in cultured cells. LC_{50} value is defined as a concentration of arsenical that causes a 50% decrease in cell viability. Average LC_{50} values are shown for primary rat hepatocytes (RH), primary normal human hepatocytes (HH), normal human epidermal keratinocytes (HK), normal human bronchial epithelial cells (HB), Urotsa, SV40-transformed epithelial cells from normal human urinary bladder (HU), and for Hela cells (HL). Cells were exposed for 24 h to iAs^{III} (sodium arsenite), MAs^{III} (methylarsine oxide or methylarsine diiodide) or $DMAs^{III}$ (dimethylarsine iodide or dimethylarsine–GSH complex). Cell viability was determined by the MTT assay.

et al. (2000) who found that MAs^{III} was more toxic for Chang human hepatocytes than was iAs^{III}. Thus, trivalent methylated arsenicals that are intermediates or final metabolites of iAs are significantly more toxic for human and animal cells than the parent compounds.

METABOLISM OF MAs^{III} AND $DMAs^{III}$

The significance of trivalent methylated arsenicals in the methylation pathway for iAs was first characterized by Cullen et al. (1984) using data from metabolic experiments in fungi and bacteria. In their scheme, iAs^{III}, MAs^{III} and $DMAs^{III}$ were intermediates formed by the reduction of their pentavalent precursors (Scheme 1) and these trivalent species were postulated to be substrates for arsenic methyltransferase(s). Consistent with this scheme, trivalent arsenicals were found to be preferred substrates for the methylation reactions in an in vitro methylating system containing rat liver cytosol (Styblo et al., 1995b). In this system, the conversion of iAs^{III} to MAs and DMAs was much faster than was conversion of iAs^{V}. Similarly, the rate of the methylation of MAs^{III} to DMAs was significantly greater than that of MAs^{V}. In addition, arsenic methyltransferase purified from rabbit liver (Zakharyan et al., 1995) has a greater affinity for MAs^{III} than for MAs^{V} (Zakharyan et al., 1999a). These data suggest that pentavalent arsenicals must be reduced in order to undergo the next methylation step. GSH, a major intracellular low molecular-weight thiol and a potent reducing agent, is thought to provide reducing equivalents for this reduction. GSH has been shown to donate electrons for the reduction of As^{V} to As^{III} in aqueous solutions (Scott et al., 1993; Delnomdedieu et al., 1994a) and in isolated rabbit



Scheme 1. Metabolic conversions of iAs in humans. GSH, reduced form of glutathione; GSSG, glutathione disulfide (the oxidized form of glutathione); AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; R, As^{V} reductase; M, As methyltransferase.

erythrocytes (Delnomdedieu et al., 1994b, 1995). Addition of GSH into in vitro systems containing rat liver cytosol promoted the synthesis of MAs and DMAs from iAs (Buchet and Lauwerys, 1985, 1988; Styblo et al., 1996a). GSH as well as other thiols supported methylation of iAs and MAs by purified arsenic methyltransferase (Zakharyan et al., 1995). It is unknown at this time whether GSH alone is responsible for the in vivo conversion of pentavalent arsenicals to trivalency. Reports by Zakharyan and Aposhian (1999b) and Radabaugh and Aposhian (2000) suggest that in mammalian tissues the reductions of As^V species are catalyzed by iAs^V and MAs^V reductases. The tissue distribution of MAs^V reductase activity in the golden hamster has been characterized by Sampayo-Reyes et al. (2000). In this animal species, the highest MAs^V reductase activity was found in the brain and the lowest in testes. The relative contributions of the GSH-dependent and enzymatically-catalyzed reactions to the overall production of trivalent arsenicals in tissues have not been determined.

Although the chemical theory of arsenic methylation postulates trivalent methylated arsenicals to be critical intermediary metabolites of iAs, there was until recently no direct evidence of their formation in human or animal tissues. Analytical techniques used for

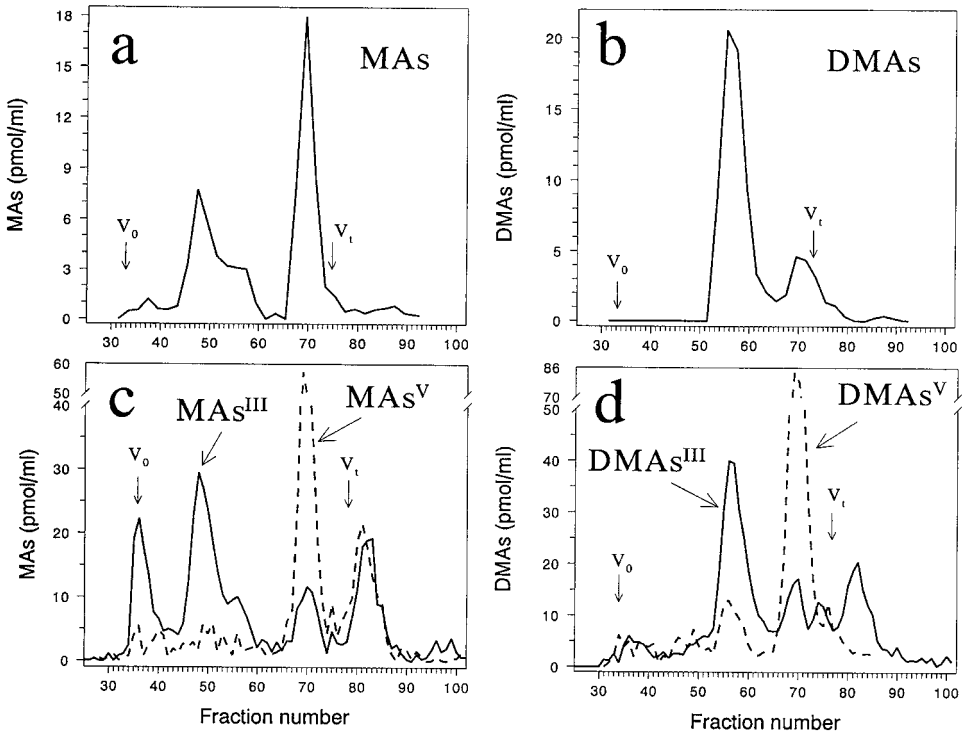


Fig. 2. Protein binding profiles for methylated arsenic metabolites in rat liver cytosol incubated in vitro with iAs^{III} (a,b) and binding profiles for custom synthesized tri- and pentavalent MAs and DMAs (c,d). (a,b) Rat liver cytosol was incubated at 37°C with 1 μM [^{73}As]iAs^{III} in the presence of GSH and *S*-adenosylmethionine for 10 (a) or 30 (b) min. Soluble cytosolic proteins were then separated on a Sephacryl S300 (50/1 cm) column. Radiolabeled metabolites in chromatographic fractions were analyzed by TLC. (c,d) Rat liver cytosol was incubated at 0°C with 1 μM ^{14}C-labeled MAs^V, MAs^{III}, $DMAs^V$ or $DMAs^{III}$ in the presence of GSH and *S*-adenosylmethionine for 30 min. Radioactivity in chromatographic fractions was determined by liquid scintillation. V_0 marks the void volume and V_t the total volume of the column.

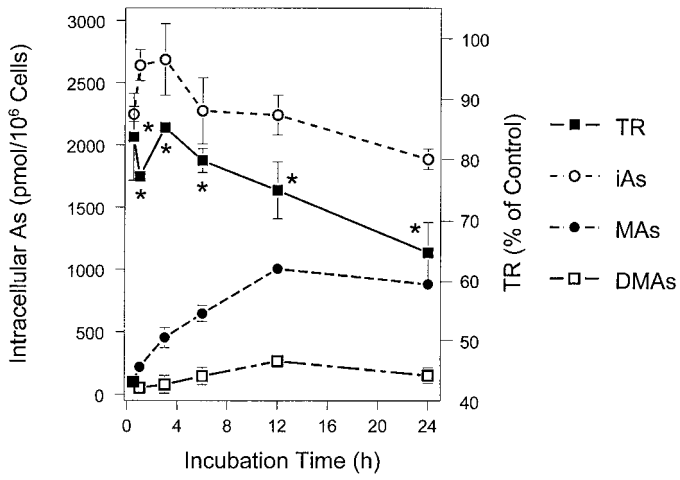


Fig. 3. Time profiles for thioredoxin reductase (TR) activity and intracellular concentrations of arsenic metabolites in primary rat hepatocytes exposed to 10 μM iAs^{III} for 24 h (mean ± SD, $n = 3$). * TR activity in treated cells is significantly different ($P < 0.05$) from that in control (untreated) cells.

arsenic speciation in biological samples routinely determine iAs^{V} and iAs^{III} but cannot speciate tri- and pentavalent methylated arsenicals. Thus, only data on total MAs (i.e., $MAs^{III} + MAs^{V}$) and total DMAs (i.e., $DMAs^{III} + DMAs^{V}$) have been commonly available to evaluate exposures to iAs and the ability of organisms to metabolize iAs. Nevertheless, indirect evidence in favor of the existence of trivalent methylated arsenicals in biological samples has been provided by recent studies. In 1996, we have reported that trivalent arsenicals (iAs^{III}, MAs^{III} and $DMAs^{III}$), unlike their pentavalent analogs, exhibit high binding affinities towards proteins (Styblo et al., 1996b). In a rat liver cytosol-containing in vitro system that methylated iAs^{III}, a significant portions of methylated metabolites produced from iAs^{III} were bound to several protein fractions (Styblo and Thomas, 1997). We examined binding affinities of these fractions by incubating them with custom-synthesized ^{14}C-labeled tri- and pentavalent MAs and DMAs. The comparison of the protein binding profiles for methylated metabolites produced from iAs^{III} with profiles for ^{14}C-standards suggested that some of the protein fractions preferably bound trivalent methylated arsenicals (Fig. 2). The results of this experiment clearly shows that the metabolism of iAs^{III} in this in vitro system yielded both trivalent and pentavalent methylated metabolites. In addition, we have detected protein bound methylated metabolites in the liver and kidney of a mouse injected with a trace dose of iAs^{III} (Styblo et al., 1996b), indicating that MAs^{III} and $DMAs^{III}$ were also products of in vivo metabolism of iAs^{III}.

Other indirect evidence for the production of trivalent methylated metabolites in the course of iAs methylation was obtained in experiments with primary cultures of rat hepatocytes exposed to 10 μM iAs^{III} for 24 h (Lin et al., 2000). This study examined the relationship between the metabolism of iAs and changes in the TR activity. Fig. 3 shows the time profiles for intracellular concentrations of iAs, MAs, DMAs and for TR activity. TR activity in treated cells was steadily decreasing between 30 min and 24 h. The intracellular concentration of iAs also decreased after reaching a peak at 1–3 h. In contrast, intracellular concentrations of DMAs and especially MAs increased steadily during the first 12 h. Regression analysis showed that the decrease in TR activity strongly correlated ($P < 0.05$) with the increase in the intracellular concentrations of MAs and DMAs, but not with the concentration of iAs (Fig. 4). Because MAs^{V} and either trivalent or pentavalent DMAs are weak inhibitors of TR (Lin et al., 1999), it is likely that the inhibition of TR activity in rat hepatocytes exposed to iAs^{III} was mostly due to the formation and accumulation of MAs^{III}.

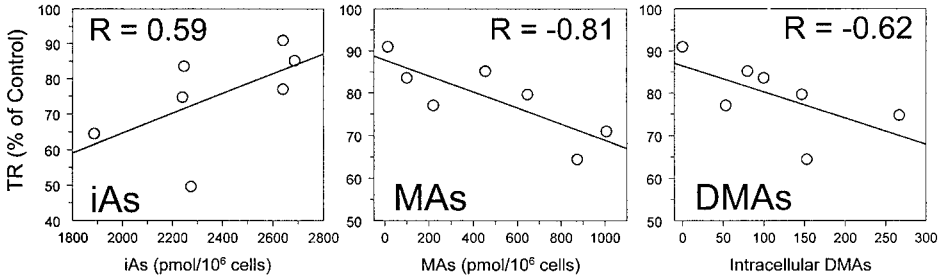


Fig. 4. Correlation between thioredoxin reductase (TR) activity and intracellular concentrations of iAs, MAs, and DMAs in rat hepatocytes exposed to 10 μM iAs^{III} for 24 h.

It is likely that interactions of trivalent arsenic metabolites with proteins and other cellular constituents are responsible for retention and toxic effects of arsenic in tissues of animals and humans exposed to iAs. However, a comprehensive evaluation of the amount and composition of arsenic metabolites retained in tissues has been limited by a paucity of analytical data. The first and only speciation analysis in animal tissues was carried out by Kenyon and associates in lung, liver and kidney of mice given a single oral dose of 1, 10, 30 or 100 μM iAs^{III}/kg (Kenyon et al., 2000). These authors found MAs and DMAs in all three organs with highest levels of DMAs in the lung and kidney. MAs represented up to 20% of the arsenic retained in tissues. Benramdane et al. (1999) have recently reported a case of a 28-year-old man who was fatally poisoned by arsenic trioxide. Significant fractions of arsenic retained in tissues of this man were represented by MAs (10–30%) and DMAs (4–19%). The highest concentrations of these metabolites were found in the liver. Recent reports by Aposhian et al. (1997, 2000b) found methylated arsenicals to be retained in tissues of individuals chronically exposed to iAs from drinking water. In their studies, treatments of arsenic-exposed individuals with an arsenic chelator, 2,3-dimercaptopropane-1-sulfonic acid (DMPS), resulted in a massive release of MAs, including MAs^{III}, in urine, suggesting that DPMS mobilized tissue depots of this metabolite.

Thus, the work of several independent laboratories has provided strong but indirect evidence for the production and retention of trivalent methylated arsenicals, MAs^{III} and $DMAs^{III}$, in cultured cells and in tissues of animals and humans exposed to iAs. However, acquiring direct evidence was possible only after optimization of analytical techniques that would be capable of the speciation of tri- and pentavalent methylated arsenicals in biological matrices.

ANALYSIS OF MAs^{III} AND $DMAs^{III}$ IN BIOLOGICAL MATERIAL

Responding to a growing demand for more powerful speciation techniques, several analytical methods have recently been developed that can provide complete speciation analyses of arsenic metabolites, including iAs^{III}, iAs^{V}, MAs^{III}, MAs^{V}, $DMAs^{III}$, and $DMAs^{V}$, in biological matrices. The method of Le and associates includes HPLC separation of arsenic species with subsequent determination of arsenic in chromatographic fractions by hydride generation–atomic fluorescence spectrometry (HG–AFS) (Le et al., 2000a). Using this method, one of the two trivalent methylated metabolites, MAs^{III}, has been found in urine from humans chronically exposed to iAs who were treated with DMPS (Aposhian et al., 2000b; Le et al., 2000b). Another study also found MAs^{III} in urine from individuals who consumed water contaminated with iAs and who were not treated with an arsenic chelator (Aposhian et al., 2000b). Differential extraction of trivalent and pentavalent arsenicals with

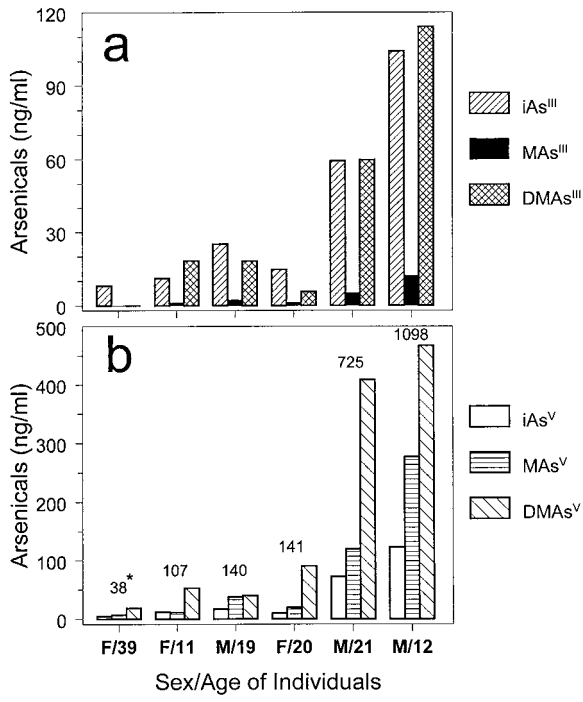


Fig. 5. Trivalent (a) and pentavalent (b) arsenic metabolites in fresh urine collected from six residents of Zimapan region (Hidalgo province), Mexico, who are chronically exposed to iAs from drinking water. Samples were analyzed using the pH-specific HG–AAS technique. Each bar represents an average of duplicates. * Numbers above bars in part b represent the sums of arsenic metabolites (ng As/ml) from part a and b for each individual.

subsequent ion-exchange chromatography has been used by Sampayo-Reyes et al. (2000) to determine hepatic contents of MAs^{III} and DMAs^{III} in golden syrian hamsters 15 h after i.p. injection of iAs^{V} (2 mg As/kg body weight). Using an optimized HPLC separation, MAs^{III} has also been found in the bile of rats during 2 h after i.v. injection of 50 μM/kg of either iAs^{III} or MAs^{III} (Gregus et al., 2000).

Another speciation technique has been developed by Del Razo and associates. This method is based on the pH-specific generation of hydrides from tri- and pentavalent iAs, MAs and DMAs with subsequent separation and detection of corresponding arsines by atomic absorption spectrometry (HG–AAS) (Del Razo et al., 2001). Reduction with borohydride at pH <2 generates arsines from either tri- or pentavalent arsenicals. At pH 6, arsines can be generated only from trivalent species. Using this method, both MAs^{III} and DMAs^{III} have recently been found in fresh urine of residents from arsenic endemic areas in Mexico (Del Razo et al., 2001). The amounts of MAs^{III} and DMAs^{III} in urine varied among individuals and were proportional to the total urinary arsenic (Fig. 5). However, trivalent methylated metabolites, particularly DMAs^{III}, have been found to be unstable in human urine and are quickly oxidized to pentavalent MAs^{V} and DMAs^{V}. The pH-specific HG–AAS analysis has also been used in our laboratory to speciate arsenic metabolites in HepG2 (human hepatocellular carcinoma) cells exposed to 0.1, 1 or 10 μM of iAs^{III}. Both MAs^{III} and DMAs^{III} were detected in cultured cells and/or in culture medium after a 24-h exposure (Fig. 6). Results of these analyses suggest DMAs^{III} to be a product of the methylation of iAs^{III} in HepG2 cells, regardless of the level of exposure. In contrast, MAs^{III} was found only in cultures exposed to 1 or 10 μM iAs^{III}. The presence of MAs^{III} and DMAs^{III} in the medium of HepG2 cells exposed to iAs^{III} suggests that these toxic metabolites could be released in vivo from hepatic cells and distributed via the blood stream throughout the human body.

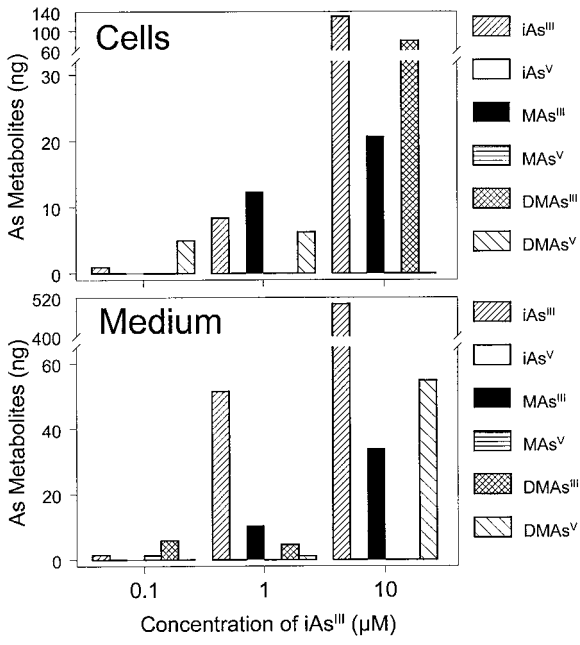


Fig. 6. Trivalent and pentavalent metabolites in HepG2 cell cultures exposed to 0.1, 1 or 10 μM iAs^{III} for 24 h. Cultured cells (approx. 6×10^6 cells/sample) and medium (2 ml/sample) were analyzed using the pH-specific HG–AAS technique. Each bar represents an average of duplicates.

SUMMARY

The generally accepted hypothesis that views biomethylation of iAs as a major detoxification mechanism has recently been challenged by experimental work of several laboratories. The results of this work have demonstrated that:

- The reduction of pentavalent methylated arsenicals in the course of arsenic metabolism yields trivalent methylated species, MAs^{III} and $DMAs^{III}$, that are more potent enzyme inhibitors and more potent cytotoxins than either iAs^{V} or iAs^{III}.
- MAs^{III} and $DMAs^{III}$ are likely to be species that are retained in tissues through interactions with specific protein targets. The protein binding of MAs^{III} and $DMAs^{III}$ may be responsible for toxicity of these metabolites or may be a detoxification mechanism.
- These toxic metabolites, MAs^{III} and $DMAs^{III}$, are generated by human hepatic cells and may be exported from the liver to other tissues.
- MAs^{III} and $DMAs^{III}$ are found in urine from humans chronically exposed to iAs from drinking water. The amounts of urinary MAs^{III} and $DMAs^{III}$ are proportional to the total urinary arsenic, and hence to the level of the exposure.

In the light of these observations, biomethylation of iAs, a process yielding toxic trivalent methylated metabolites, appears to be a mechanism of activation of arsenic as a toxin and possibly as a carcinogen. Because of the adverse biological effects of these metabolites, the analysis of urinary MAs^{III} and $DMAs^{III}$ may serve as a useful tool for the evaluation of health risks associated with exposure to iAs.

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Speciation of Human Urinary Arsenic Including the Trivalent Methylation Metabolites

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ABSTRACT

We describe recent developments on speciation analysis of inorganic arsenic and its metabolites, monomethylarsonic acid (MMA^V), dimethylarsinic acid (DMA^V), monomethylarsonous acid (MMA^{III}), and dimethylarsinous acid (DMA^{III}), in human urine. MMA^{III} and DMA^{III} are key intermediates in the metabolic pathway of arsenic biomethylation, which involves step-wise reduction of pentavalent to trivalent arsenic species followed by oxidative addition of a methyl group. Speciation analysis was carried out using ion pair chromatographic separation of arsenic species with hydride generation atomic fluorescence detection. Speciation of arsenate (As^V), arsenite (As^{III}), MMA^V, MMA^{III}, DMA^V and DMA^{III} in human urine samples was complete in 6 min. Detection limits were in the range of 0.5–2 μg/l, allowing for direct urine analysis without any sample pretreatment. Urine samples collected from 99 people before and after a single oral administration of 300 mg sodium 2,3-dimercapto-1-propane sulfonate (DMPS) were analyzed for arsenic species. MMA^{III} was found in 207 out of 454 urine samples. DMA^{III} was detected in 9 urine samples. Urine samples collected from 10 people before the administration of DMPS also contained detectable MMA^{III}, confirming that MMA^{III} in urine samples did not necessarily arise from the reduction of MMA^V by DMPS. Both MMA^{III} and DMA^{III} were unstable in urine and were readily oxidized to MMA^V and DMA^V during sample storage. Thus, the MMA^{III} and DMA^{III} detected in the urine samples are probably underestimated. Methods for stabilizing these labile arsenic species are needed for further arsenic metabolism studies.

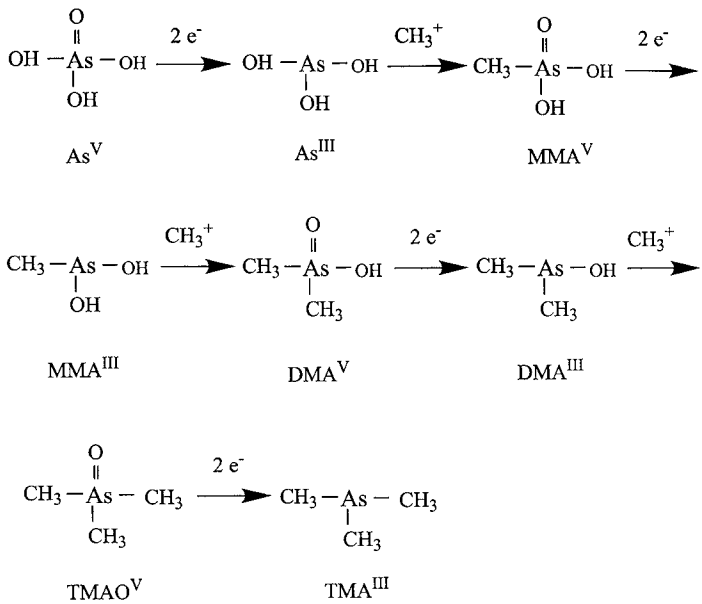
Keywords: arsenic speciation; metabolism; analytical technique; human urine; methylation metabolite

INTRODUCTION

Chronic exposure to high levels of arsenic is associated with the prevalence of skin, lung and bladder cancers (Tseng, 1977; Chen et al., 1992; Hopenhayn-Rich et al., 1996a; NRC, 1999) and is a risk factor of several other internal cancers and non-cancerous effects (Cebrian et al., 1983; Chen et al., 1985; Chatterjee et al., 1995; Hopenhayn-Rich et al., 1998; NRC, 1999; Smith et al., 1999). However, health effects from exposure to low levels of arsenic are a topic of many debates. Mechanisms of actions are not well understood (Pontius et al., 1994; Yamauchi and Fowler, 1994; Chappell et al., 1997; NRC, 1999).

Arsenic species ingested from drinking water are mainly inorganic arsenate (As^V) and arsenite (As^{III}). The inorganic arsenic species undergo metabolism in the body. Two major metabolites, dimethylarsinic acid (DMA^V) and monomethylarsonic acid (MMA^V), along with the inorganic arsenic species have been commonly observed in human urine (Buchet et al., 1981; Foà et al., 1984; Buchet and Lauwerys, 1994; Vahter, 1994; Vahter et al., 1995; Hopenhayn-Rich et al., 1996b; Le and Ma, 1998). Urinary excretion is the major pathway for the elimination of arsenic from the body (Crecelius, 1977; Freeman et al., 1979; Tam et al., 1979; Pomroy et al., 1980; Vahter, 1983; Le et al., 1993, 1994; Buchet and Lauwerys, 1994; Yager et al., 1997). Thus, speciation of arsenic in urine has been considered a measure of recent exposure to arsenic.

Speciation of arsenic has contributed much to the understanding of arsenic metabolism. It is now accepted that biomethylation is the major metabolic process for inorganic arsenic (Cullen and Reimer, 1989; Yamauchi and Fowler, 1994; Styblo et al., 1995; Goyer, 1996; Aposhian, 1997; NRC, 1999; Vahter, 1999). The stepwise methylation process is believed to involve a sequence of a two-electron reduction of arsenic followed by oxidative addition of a methyl group, as outlined in Scheme 1 (Cullen et al., 1984, 1989; Cullen and Reimer, 1989). $TMAO^V$ (trimethylarsine oxide) and TMA^{III} (trimethylarsine) are the end product



Scheme 1. Biomethylation of arsenic showing a sequence of reduction of pentavalent to trivalent arsenic followed by oxidative addition of a methyl group. (Reprinted with permission from Le et al., 2000a.)

produced by some microorganisms. DMA^V is the usual end product detected in humans until recently when DMA^{III} is also detected in human urine (Le et al., 2000a; Mandal et al., 2001; Styblo et al., 2001).

Most previous studies have focused on the speciation of relatively stable arsenic compounds that are readily detected in human urine (Crecelius, 1977; Buchet et al., 1981; Foà et al., 1984; Yamauchi et al., 1992; Hakala and Pyy, 1995; Vahter et al., 1995; Hopenhayn-Rich et al., 1996b; Del Razo et al., 1997; Ng et al., 1998; Vahter, 1999). However, the determination of the intermediate metabolites, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), is also important to the understanding of the arsenic methylation pathway (Cullen and Reimer, 1989; Aposhian et al., 1999; Styblo et al., 1999; Zakharyan et al., 1999; Sampayo-Reyes et al., 2000). Furthermore, recent studies have shown that the trivalent arsenic methylation metabolites are as toxic as or even more toxic than the inorganic arsenic species (Petrick et al., 2000; Styblo and Thomas, 1995; Styblo et al., 1997; Lin et al., 1999; Petrick et al., 2001; Mass et al., 2001). Thus, there has been much interest in the determination of these methylation intermediary metabolites in humans. This paper describes our recent development in the speciation of arsenic metabolites including the intermediates in human urine.

METHODOLOGY

Instrumentation

Separation of various arsenic species was carried out using an high performance liquid chromatography (HPLC) system that consisted of an HPLC pump (Model 307, Gilson, Middletone, WI), a 6-port sample injector with a 20-μl sample loop (Model 7725i, Rheodyne, Rohnet Park, CA), and an appropriate column (Le and Ma, 1998). The column was mounted inside a column heater (Model CH-30, Eppendorf, Westbury, NY) which was controlled by a temperature controller (Model TC-50, Eppendorf). The column temperature was maintained at $50 \pm 1^{o}C$. Mobile phase was pre-heated to the temperature of the column by using a precolumn coil of 50 cm stainless steel capillary tubing, which was also placed inside the column heater.

A hydride generation atomic fluorescence spectrometer (HGAFS) (Model Excalibur 10.003, P.S. Analytical, Kent, UK) was used for the detection of arsenic. The combination of HPLC and HGAFS is shown schematically in Fig. 1 (Ma and Le, 1998). Effluent from the HPLC column directly meets at two T-joints, with continuous flows of hydrochloric acid (1.2 M, 10 ml/min) and sodium borohydride (1.3%, 3 ml/min) introduced by using a

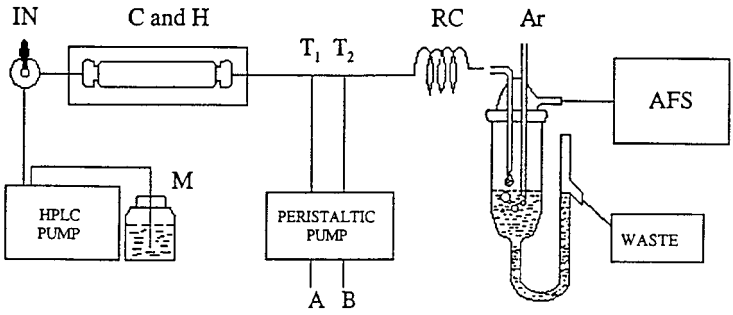


Fig. 1. A schematic diagram showing the HPLC–HGAFS system for arsenic speciation analysis. A, acid (1.2 M HCl); AFS, atomic fluorescence detector; Ar, argon carrier gas; B, 1.3% $NaBH_4$ in 0.1 M NaOH; C and H, column and column heater; IN, sample injector; M, mobile phase; RC, reaction coil (50 cm long and 0.8 mm i.d.); T1 and T2, T-joints. (From Ma and Le, 1998. Reprinted with permission.)

peristaltic pump. Upon mixing, arsenic hydrides (arsines), generated from the reaction, are separated from liquid waste in a gas/liquid separator apparatus and carried by a continuous flow of argon carrier gas (250 ml/min) to the atomic fluorescence detector. The atomic fluorescence detector consisted of a boosted discharge arsenic hollow cathode lamp as an excitation source, a hydrogen diffusion flame to atomize arsenic, and fluorescence detection optics. The hydrogen, produced as a hydride generation by-product, was sufficient to maintain the hydrogen diffusion flame that decomposes arsines and atomizes arsenic. Atomic fluorescence from arsenic was detected at a right angle using a solar blind photomultiplier tube. A Pentium computer with chromatography software (Star Workstation, Varian, Victoria, Australia) was used to record and process signals from the atomic fluorescence detector. An integrator (Model 3390A, Hewlett Packard, Boise, Idaho) with both peak area and peak height measurement capabilities was also used to record chromatograms.

Standards, Reagents, and Samples

An atomic absorption arsenic standard solution containing 1000 mg As/l as arsenite (Sigma, St. Louis, MO) was used as the primary arsenic standard. Sodium arsenate, $As(O)OH(ONa)_2 \cdot 7H_2O$) and sodium cacodylate, $(CH_3)_2As(O)ONa$ were obtained from Sigma, and monomethylarsonate, $CH_3As(O)OHONa$, was obtained from Chem Service (West Chester, PA). The source of MMA^{III} was the solid oxide (CH_3AsO), and DMA^{III} was the iodide [$(CH_3)_2AsI$], which were prepared following literature procedures (Burrows and Turner, 1920; Cullen et al., 1989). Trimethylarsine oxide [$(CH_3)_3AsO$, TMAO] was synthesized following the procedures of Merijanian and Zingaro (1966). Solutions of standard arsenic compounds were prepared by appropriate dilutions with deionized water from 1000 mg/l stock solutions. Solutions of MMA^{V}, MMA^{III}, DMA^{III}, DMA^{V} and TMAO were standardized against a primary As^{III} standard using an inductively coupled plasma mass spectrometer (Le and Ma, 1998; Feldmann et al., 1999; Le et al., 1996, 2000a).

Tetrabutylammonium hydroxide (TBAH), malonic acid, and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Aldrich (Milwaukee, WI). HPLC grade methanol was from Fisher (Pittsburgh, PA). The HPLC mobile phase solutions were prepared in deionized water and filtered through a 0.45-μm membrane prior to use. Sodium borohydride (Aldrich) solutions (1.3%) in 0.1 M sodium hydroxide (Fisher) were prepared fresh daily. All reagents used were of analytical grade or better.

A standard reference material (SRM), Toxic Metals in Freeze-Dried Urine SRM 2670, was obtained from National Institute of Standards and Technology (NIST, Gaithersburg, MD). The freeze-dried urine was reconstituted by the addition of 20.0 ml of deionized water as recommended by the supplier.

Urine samples were collected from 41 people in Inner Mongolia, China (Aposhian et al., 2000a) and 58 people in Romania (Aposhian et al., 2000b). Arsenic levels in their well water that was used for consumption were 510–660 μg/l for the Chinese group (Aposhian et al., 2000a) and 3–161 μg/l for the Romanian group (Aposhian et al., 2000b). They were asked to exclude seafood consumption for 3 days prior to and during the urine sample collection period. They stopped drinking well water and were provided with distilled water to drink. They were fasted overnight and then orally administered 300 mg of sodium 2,3-dimercapto-1-propane sulfonate (DMPS). A urine sample was collected from each participant before the administration of DMPS. Three urine samples were collected 0–2, 2–4, and 4–6 h after the administration of DMPS. Sample pH was adjusted to 4–5.

Urine samples were collected in 3-l polyethylene containers (Baxter Laboratories, Inc., Morton, IL), and the containers were then placed in portable iceboxes containing dry ice. The samples were kept frozen during transportation and were stored at −20°C until before the analysis when the samples were thawed at room temperature and an aliquot (20 μl) was analyzed for arsenic species using HPLC–HGAFS.

Speciation of As^{III}, As^{V}, MMA^{V} and DMA^{V} in Urine

Analyses of arsenic speciation in urine samples were carried out by using HPLC–HGAFS. An aliquot of a sample was filtered through a 0.45-μm membrane prior to HPLC–HGAFS analysis. A reversed-phase column (ODS-3, 150 × 4.6 mm, 3 μm particle size, Phenomenex, Torrance, CA) was used for separation. Mobile phase contained 5 mM tetrabutylammonium hydroxide, 4 mM malonic acid and 5% methanol (pH 5.8), and its flow rate was 1.5 ml/min.

Speciation of As^{III}, As^{V}, MMA^{V}, DMA^{V}, MMA^{III}, and DMA^{III} in Urine

A reversed-phase column (ODS-3, 150×4.6 mm, 3 μm particle size, Phenomenex) was used for separation. A mobile phase solution (pH 5.9) contained 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol, and its flow rate was 1.2 ml/min. The concentrations of hydrochloric acid (1.2 M) and sodium borohydride (1.3%) for hydride generation were optimized for maximum sensitivity of the less abundant arsenic species in urine, such as MMA^{III}, DMA^{III}, and MMA^{V}. Arsines generated were separated from liquid waste and carried by a continuous flow of argon to the atomic fluorescence detector for quantitation.

Determination of TMAO

An anion-exchange column (PRP X-100, 150 × 4.1 mm, Hamilton, Reno, NV) was used to separate TMAO with a mobile phase containing 5 mM phosphate (pH 8.2) and 5% methanol. The flow rate of the mobile phase was 1.0 ml/min.

RESULTS AND DISCUSSION

Speciation of the Usual Arsenic Compounds in Human Urine

We have previously investigated HPLC conditions and optimized them for rapid separation of As^{III}, As^{V}, MMA^{V} and DMA^{V} (Le and Ma, 1998). The speciation of these four usual arsenic compounds in urine is complete in 4 min (Fig. 2). The efficient separation within a short time was achieved by combining the use of a shorter HPLC column (15 cm) packed with smaller particles (3 μm) and the use of faster flow rate (1.5 ml/min).

The efficiency of a HPLC column is inversely proportional to the square of packing material diameter (Chen and Horvath, 1995; Snyder and Kirkland, 1977). Therefore, separation can be improved by using columns packed with smaller particles. Conventionally, 5–10 μm particle size is commonly used in HPLC columns. We used columns packed with 3-μm particles, and the improvement of separation efficiency enabled us to use shorter columns for the speciation of arsenic.

Furthermore, the optimum flow rate of the mobile phase is higher when a smaller size of particle is used in HPLC column packing. This is governed by the Van Deemter equation (Snyder and Kirkland, 1977; Chen and Horvath, 1995). A higher flow rate results in a faster HPLC separation.

Detection limits for arsenic species in urine matrix were 0.5 μg/l for As^{III} and MMA^{V} and 1 μg/l for As^{V} and DMA^{V}. They were measured as arsenic concentrations corresponding to three times the signal-to-noise ratio. This detection capability allows for direct speciation of arsenic in human urine samples from the general population. Examples of the reported concentrations of arsenic in human urine from the general population are (mean ± standard deviation, μg/l) 9 ± 7 from a U.S. population (Kalman et al., 1990), 17 ± 11 and 11 ± 6 from European studies (Foà et al., 1984; Buchet et al., 1996), 21 ± 7 from Taiwan (Lin and Huang, 1995), and 121 ± 101 from Japan (Yamauchi et al., 1989). People exposed to higher levels of arsenic from drinking water and food have corresponding higher levels of urinary arsenic, e.g. 56 ± 13 from Blackfoot disease patients (Lin and Huang, 1995), 274 ± 98 from a highly exposed Argentina population (Vahter et al., 1995), and 450–700

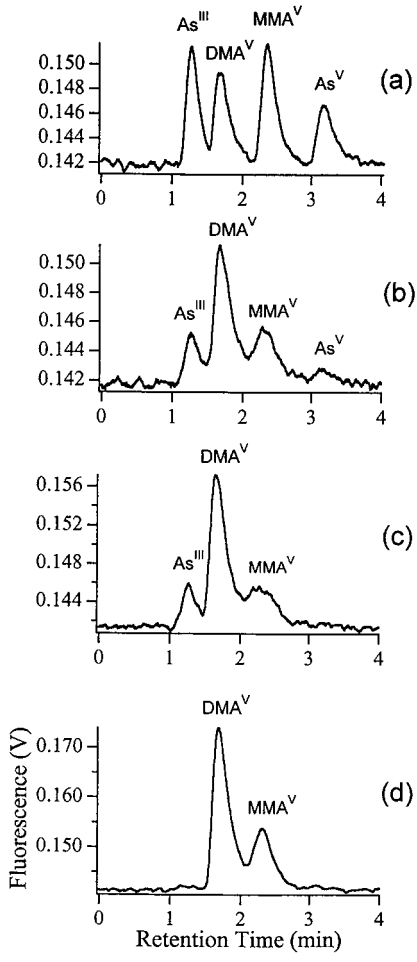


Fig. 2. HPLC–HGAFS analyses of As^{III}, As^{V}, MMA^{V}, DMA^{V} in a standard solution (a), a volunteer urine samples (b,c), and a standard reference material urine (d). A reversed-phase column (Phenomenex ODS-3, 15 cm × 4.6 mm, 3 μm particle size) was used for ion paring separation. Mobile phase contained 5 mM tetrabutylammonium hydroxide, 4 mM malonic acid and 5% methanol (pH 5.8), and its flow rate was 1.5 ml/min. The HPLC column temperature was maintained at 50°C.

from a highly exposed Mexican group (Del Razo et al., 1997). Typical chromatograms from the analyses of urine samples are shown in Fig. 2b,c. These samples were from two volunteers who did not have excess exposure to arsenic and who refrained from eating any seafood for 3 days prior to the collection of the first morning void. Concentrations of arsenic species in urine sample (b) were 4, 15, 4, and 2 μg/l for As^{III}, DMA^{V}, MMA^{V}, and As^{V}, respectively. Concentrations of arsenic species in urine sample (c) were 3, 23, and 3 μg/l for As^{III}, DMA^{V}, and MMA^{V}, respectively. As^{V} in urine sample (c) was below detection limit.

A standard reference material, SRM 2670 (from NIST, MD), was used for method validation and quality control purpose. This SRM consists of two components, normal level and elevated level of toxic metals. Fig. 2d shows a chromatogram from the analysis of the SRM 2670 urine containing normal levels of toxic metals. Results for the speciation of arsenic in this SRM using the HPLC–HGAFS method are 49 ± 5 for DMA^{V} and 11 ± 3 for MMA^{V}. A reference value of total arsenic in this SRM is 60 μg/l. The other component of the SRM contains elevated levels of toxic metals and the certified value for total arsenic

concentration is 480 ± 100 μg/l. Results for the speciation of arsenic using the HPLC–HGAFS method are 46 ± 5 for DMA^V, 11 ± 3 for MMA^V, and 460 ± 25 for As^V. These results are in good agreement with the certified and literature values (Crecelius and Yager, 1997).

Speciation of Intermediate Arsenic Metabolites in Human Urine

The method described above is suitable for the rapid speciation of As^{III}, As^V, MMA^V and DMA^V (Le and Ma, 1998) and has been demonstrated in pilot epidemiological studies of arsenic exposure and health effects (Calderon et al., 1999). We initially attempted to adopt this method for the speciation of MMA^{III} and DMA^{III} in human urine. However, MMA^{III} coeluted with As^{III} and DMA^V, and DMA^{III} overlapped with As^V within the narrow separation time window (4 min) (Le et al., 2000b). To obtain a separation of MMA^{III} and DMA^{III}, we modified HPC separation conditions to allow for a wider separation window. This was achieved by adjusting malonic acid concentration in the HPLC mobile phase. By reducing malonic acid concentration to 3 mM and a slight adjustment of the pH and flow rate of the mobile phase, an extended separation time period was obtained (Fig. 3). The modified chromatography allowed for the separation of MMA^{III} from As^{III} and DMA^V, and the separation between DMA^{III} and As^V (Fig. 3). This is at the expense of a longer retention time for the arsenic species; the speciation of the six arsenic compounds, As^{III}, As^V, MMA^V, DMA^V, MMA^{III}, and DMA^{III} is complete in 6 min (Fig. 3).

Fig. 3 also shows chromatograms obtained from the analyses of a urine sample collected 4 h after a person ingested 300 mg DMPS (dotted traces). Co-injection of the urine sample with authentic MMA^{III} standard demonstrates the co-elution of the suspected MMA^{III} in the sample with that of the standard MMA^{III} (Fig. 3b), confirming the identity of MMA^{III} in the urine sample. Similarly, co-injection of the urine sample with standard DMA^{III} (Fig. 3c) confirms the presence of DMA^{III} in the sample.

We detected MMA^{III} in 207 samples and DMA^{III} in 9 samples out of 454 samples from 99 subjects. The highest MMA^{III} concentration in the urine samples was 240 μg/l. In most cases, MMA^{III} and DMA^{III} were found in urine samples collected after the administration of DMPS. However, 10 samples collected before the administration of DMPS also contained detectable MMA^{III} (Aposhian et al., 2000b). Fig. 4 shows chromatograms from the speciation analysis of such a urine sample (Fig. 4a) and the sample spiked with MMA^{III} (Fig. 4b). The urine sample was collected before the administration of DMPS. MMA^{III} is clearly present in the urine sample from the subject who was not administered DMPS. Two samples collected before the administration of DMPS also contained detectable DMA^{III}.

Furthermore, analyses of some urine samples containing added DMPS (438 μM), MMA^V (0.3 μM), and DMA^V (0.7 μM) did not show evidence of MMA^{III} and DMA^{III} formation (Le et al., 2000a). These results demonstrate that the MMA^{III} and DMA^{III} detected in human urine samples are not due to endogenous reduction of MMA^V and DMA^V by DMPS.

The most commonly used hydride generation methodology does not distinguish between pentavalent and trivalent arsenic species unless carried out at selective pH conditions. The pentavalent arsenic species (As^V, MMA^V, DMA^V, and $TMAO^V$) require a low pH (usually pH <1) to form hydride upon treatment with sodium borohydride. At a higher pH (>5), only trivalent arsenic species (As^{III}, MMA^{III}, and DMA^{III}) form volatile hydrides.

Determination of TMAO

Trimethylarsine oxide (TMAO) is an expected product of methylation of DMA^{III}, as shown in Scheme 1. We probed the presence of TMAO to examine whether DMA^{III} is further methylated to TMAO in humans. Under the ion pair chromatographic conditions described above, TMAO coeluted with As^{III} and thus, could not be differentiated. Thus, we

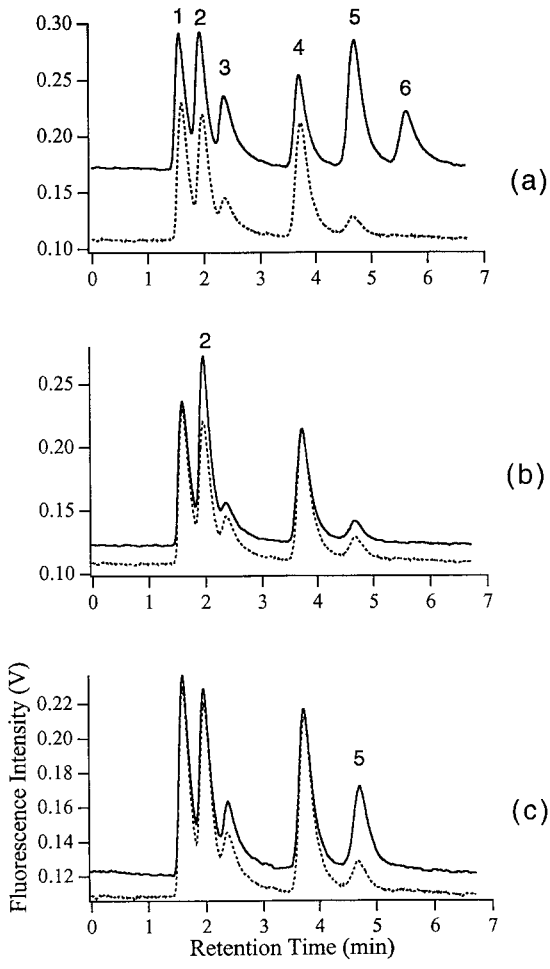


Fig. 3. Chromatograms obtained from the HPLC–HGAFS analyses of As^{III}, As^{V}, MMA^{V}, DMA^{V}, MMA^{III}, and DMA^{III} in deionized water (a, solid trace), a urine sample (dotted traces) and the urine sample spiked with MMA^{III} (b, solid trace), and DMA^{III} (c, solid trace). Separation was carried out on an ODS-3 column (15 cm × 4.6 mm, 3 μm particle size) with a mobile phase (pH 5.9) containing 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol. The flow rate of the mobile phase was 1.2 ml/min. The column temperature was 50°C. Peaks labeled 1–6 correspond to As^{III}, MMA^{III}, DMA^{V}, MMA^{V}, DMA^{III}, and As^{V}, respectively. The urine sample was collected from a person 4 h after the administration of 300 mg DMPS. For clarity, chromatograms in solid traces were manually shifted on vertical axis. (Adapted with permission from Le et al., 2000a.)

used a different separation mode, strong anion-exchange chromatography (Fig. 5). TMAO is resolved from the other arsenicals. Using this method, we analyzed all the urine samples that contained DMA^{III}, and we did not find detectable TMAO in any of the urine samples.

Fig. 5 shows chromatograms from the speciation analysis of arsenic standards and a urine sample using strong anion-exchange separation. The urine sample was obtained from a volunteer 4 h after the administration of DMPS and it contains both MMA^{III} and DMA^{III} as shown in Fig. 3. Reanalysis of the same sample under the conditions suitable for TMAO analysis (Fig. 5a) confirms that there was no detectable TMAO in the urine sample (Fig. 5b).

TMAO has been identified as a metabolite in bacterial systems. Our recent speciation analyses of rat urine also identified TMAO (Cohen et al., 2001), consistent with previous findings (Wanabauchi et al., 1996). However, little is known about TMAO in humans. Failure to observe TMAO in human urine suggests that either its concentration in urine is below the detection limit of the method or it is further metabolized to trimethylarsine (TMA^{III}) and subsequently exhaled into the breath as it is a volatile species.

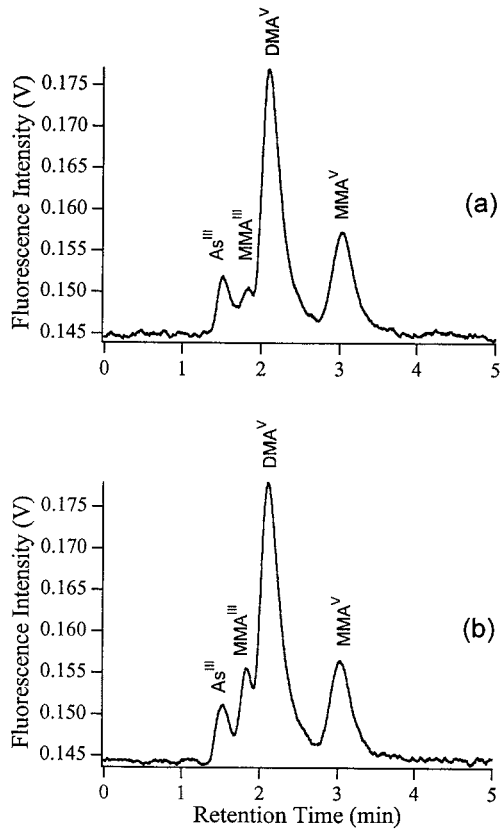


Fig. 4. Chromatograms showing speciation analyses of arsenic in a urine sample (a), and the urine sample with addition of MMA^{III} (b). The urine sample was collected from a person who was not administered with DMPS. Similar conditions as shown in Fig. 3 were used.

Stability of MMA^{III} and DMA^{III}

Both MMA^{III} and DMA^{III} were unstable in urine, although low temperature conditions (4 and −20°C) improved the stability of these arsenic species over the room temperature storage condition (Gong et al., 2001). MMA^{III} in deionized water was relatively stable for nearly 4 months, when stored at 4 or −20°C; less than 10% of MMA^{III} in deionized water was oxidized to MMA^{V}. In contrast, majority of MMA^{III} in urine was oxidized to MMA^{V} after 1 month at 4 or −20°C. DMA^{III} in deionized water was stable for only 2–3 days, after which it was oxidized to DMA^{V}. DMA^{III} in urine was completely oxidized to DMA^{V} within a day at 4 or −20°C. The rapid oxidation of DMA^{III} to DMA^{V} is probably why DMA^{III} has not been commonly detected in human urine samples (Foà et al., 1984; Kalman et al., 1990; Buchet and Lauwerys, 1994; Hakala and Pyy, 1995; Hopenhayn-Rich et al., 1996b; Kavanagh et al., 1998; Chappell et al., 1999; Ng et al., 1998; Vahter, 1999).

Table 1 shows the residual amounts of MMA^{III} in water and urine samples after the samples were stored for 1 month and for 4 months. MMA^{III} (100 μg/l) was spiked to the deionized water and urine samples and the samples were stored at −20, 4, or 25°C. There was no DMA^{III} detected in either deionized water or urine samples under the same storage conditions. Clearly, DMA^{III} is less stable than MMA^{III}, consistent with the fact that MMA^{III} were found in more frequent urine samples (207 out of 454), whereas DMA^{III} was found in only a few urine samples (Aposhian et al., 2000a,b; Le et al., 2000a,b; Styblo et al., 2001 (this volume)).

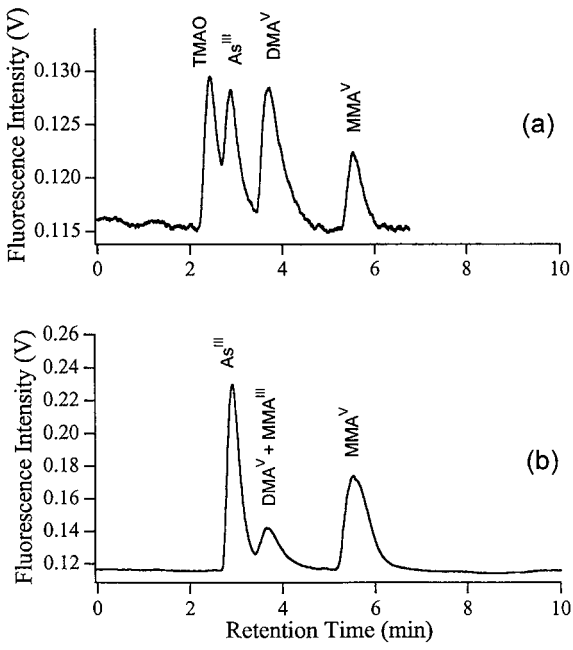


Fig. 5. Chromatograms showing HPLC–HGAFS analyses of trimethylarsine oxide (TMAO) and a urine sample collected 4 h after the administration of DMPS. Separation was carried out on a strong anion exchange column (PRP X-100, 15 cm × 4.1 mm) with a mobile phase containing 5 mM phosphate (pH 8.2) and 5% methanol. Note that TMAO was not detectable in the urine sample.

TABLE 1

Residual amounts of MMA^{III} (μg/l) in water and urine samples after each sample was spiked with 100 μg/l MMA^{III} and stored for up to 4 months

Storage temperature (°C)	MMA^{III} in water		MMA^{III} in urine	
	1 month	4 months	1 month	4 months
−20	90 ± 5	90 ± 5	38 ± 3	4 ± 2
4	100 ± 5	93 ± 5	5 ± 2	n.d.
25	5 ± 2	5 ± 1	n.d.	n.d.

n.d., below detection limit of 2 μg/l.

It is conceivable that MMA^{III} and DMA^{III} species are partially oxidized to MMA^{V} and DMA^{V} during sample collection, handling, and storage. The MMA^{III} and DMA^{III} detected in the urine samples may represent the residual amounts of these trivalent arsenic species. It is not surprising that they have not been often detected in human urine samples, especially when some analytical techniques of poorer detection limits were used. Methods for stabilizing these species need to be developed.

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Arsenic Exposure and Health Effects IV
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Dose–Response Relationships for the Metabolism and Urinary Excretion of Arsenicals in Humans

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ABSTRACT

Two population-based studies of the metabolism and fate of arsenic were performed in individuals who chronically consumed drinking water that contains inorganic arsenic. The first study examined the relationship between the intensity of exposure to inorganic arsenic in drinking water and the urinary excretion of the methylated metabolites, methyl arsenic and dimethyl arsenic. The output of methyl and dimethyl arsenic in urine was found to be relatively constant throughout the day and over a 5-day period. Over a wide range of exposure to inorganic arsenic in drinking water (8–620 μg/l), neither the amount nor percentage of total arsenic in urine that was present as methyl or dimethyl arsenic declined. That is, capacities to methylate inorganic arsenic and to excrete its metabolites in urine were not exceeded over this exposure range. Percentages of the total arsenic in urine that were accounted for by each methylated metabolite varied among individuals. Inter-individual variation could arise from polymorphisms in the enzyme that methylates arsenicals. Further characterization of the enzymology of arsenic methylation in humans will be required to identify sources of interindividual variation. The second study was a pilot study of arsenic and selenium status in chronically exposed individuals. Selenium modifies the distribution, metabolism, retention, and toxicity of inorganic arsenic in many experimental systems; however, few data are available on arsenic and selenium interactions in humans. Although the concentrations of arsenic in blood and urine were correlated, the concentrations of selenium in blood and urine were not. This difference may reflect homeostatic control of selenium metabolism and the absence of such control for arsenic metabolism. When expressed on the basis of body mass, the concentrations of arsenic and selenium in blood, but not in urine, were significantly correlated. Body mass has previously been identified as potential confounder of the analysis of dose–response relationships in individuals chronically exposed to inorganic arsenic.

Keywords: arsenic; methyl arsenic; dimethyl arsenic; selenium; blood; serum; urine; human

INTRODUCTION

Humans, like many other species, are efficient methylators of As. Ingestion or inhalation of inorganic As (iAs) precedes the appearance of iAs, methyl As (MAs) and dimethyl As (DMAs) in urine (Crecelius, 1977; Smith et al., 1977; Yamauchi and Yamamura, 1979). The methylation of As is enzymatically catalyzed (Aposhian, 1997); therefore, it is dependent on those factors that affect the rate and capacity terms of enzymatic reactions. These include the availability of cofactors and substrates required for the reaction, and variation in the kinetic properties of the enzyme that catalyzes the reaction. Variation in availability of *S*-adenosylmethionine (AdoMet), the donor of methyl groups used to methylate As, may be due to insufficient intake of dietary precursors or to genetic variation in the capacity to synthesize AdoMet. Indeed, reduced dietary intake of methionine, an AdoMet precursor, diminishes As methylation in rabbits (Vahter and Marafante, 1987). Variation in the kinetic properties of the methyltransferase catalyzing the formation of MAs and DMAs can arise from polymorphisms in the gene that encodes this protein, resulting in structural changes that alter the rate of catalysis. Furthermore, because methylation is enzymatically catalyzed, the reaction is saturable. That is, there is a concentration of precursor yielding a maximal rate for product formation; at higher precursor concentrations, the rate of conversion does not increase and the reaction is saturated. Marcus and Rispin (1988) postulated that saturation of capacity for the methylation of As results in a threshold for its action as a carcinogen. This model posits that iAs is the carcinogenic form of the element, that MAs and DMAs are inactive forms of As, and that saturation of the methylation capacity results in a rise in the concentration of iAs in tissues. Two lines of evidence suggest that this model is inadequate. First, there is little evidence from population-based studies that methylation capacity is exceeded in humans over a very wide range of exposures to iAs (Hopenhayn-Rich et al., 1993). Second, considerable evidence suggests that both MAs and DMAs exert distinct and potent cytotoxic and genotoxic effects and that methylation of As is likely to be an activation process, not a means of detoxification (for reviews, see Styblo et al., 2001 (this volume); Thomas et al., 2001). Hence, understanding the relation between exposure to iAs and the formation and excretion of methylated metabolites may be central to assessing the hazards of chronic exposure to iAs.

There is also evidence of interactions between As and Se that affect the disposition, metabolism, and toxicity of these metalloids. There are striking similarities in the metabolism of As and Se. Both undergo reductive metabolism and unique enzymes convert Se and As to methylated derivatives (Mozier et al., 1988; Zakharyan et al., 1995). Like iAs, the major urinary metabolite for Se is a methylated metabolite (trimethylselenonium ion) (Hasunuma et al., 1993). The toxicity of inorganic Se is reduced by exposure to iAs (Levander, 1977); however, iAs potentiates the toxicity of some methylated Se compounds (Kraus and Ganther, 1989). Se affects the cytotoxicity and genotoxicity of arsenicals (Ueda et al., 1997; Biswas et al., 1999) and dietary Se deficiency reduces the urinary excretion of MAs and DMAs by iAs-exposed mice (Kenyon et al., 1997, 1999). Addition of selenite to culture media of cultured primary rat hepatocytes increases the retention of iAs and decreases formation of MAs and DMAs by cells (Styblo and Thomas, 2001). Differences in the Se nutriture of various human populations exposed to iAs may at least in part account for differences among populations in the manifestations of chronic As toxicity (National Research Council, 1999). Se nutriture is a significant modifier of cancer risk in humans (Clark et al., 1998; Yu et al., 1999). It is possible that the incidences of As-induced cancers in individuals chronically exposed to iAs are affected by Se intake from food and water. Assessment of the relationship between Se nutriture and As metabolism and excretion in individuals chronically exposed to iAs could help characterize the role of an essential element in the modification of susceptibility to the chronic toxic or carcinogenic effects of iAs.

This reports summarizes recent work on the magnitude and pattern of excretion of methylated arsenicals in urine by individuals who are chronically exposed to iAs in drinking water and the results of a pilot study of biomarkers of As and Se status in chronically exposed individuals. These studies examine both dose-dependencies for the excretion of MAs and DMAs in urine and the potential interactions between the urinary excretion of As and Se. In addition, the pilot study provides novel data on the concentrations of As in blood and in urine of individuals chronically exposed to iAs in drinking water.

METHODS

Source Population and Exposure to iAs

Residents of Millard County, Utah, were recruited to participate in two studies of As exposure and excretion. The first study of urinary excretion of arsenical-containing metabolites included 95 participants from 28 families. The second study of As and Se status involved 16 subjects from six families. Participants were long-term residents of the county who consumed water from wells that yielded water that contained iAs. Use of tobacco, alcohol, and caffeine were minimal because study participants were mostly members of the Church of Jesus Christ of Latter-Day Saints. All participants had been at their current residences for at least 5 years. About half of the participants resided in Delta, Utah, where the iAs concentration in drinking water was less than 20 μg/l; the remainder resided elsewhere in Millard County and used wells with iAs concentrations exceeding 20 μg/l. If possible, all members of a family at least 8 years of age were recruited to participate in the study. Families were excluded from the study if a family member worked in the mining or smelting industries or if all family members were not available for the entire sampling period. Protocols for participation in these studies were approved by an institutional review board. All adult participants signed an informed consent form; assent for the participation of child was given by the child and a parent.

Exposure Assessment and Sample Collection

Each participant completed an exposure assessment questionnaire to provide demographic data, medical history, and length of residence in their current home. Participants were also queried on drug, alcohol, and tobacco use, on daily water consumption, and on potential exposure to As from occupational or environmental sources. All participants used a commode specimen collection system (Sage Products, Crystal Lake, IL) for urine collections. In the first study, participants started (on a Tuesday) with collection of the first urine voided upon waking (first morning void, FMV) and collected the FMV for 5 consecutive days. On 1 day of the study, each participant collected every urine voiding in a separate container. Each urine sample (up to 700 ml) was labeled with the date and time of collection, sealed in a plastic bag, and stored in a cold chest with frozen refrigerant packs. FMV samples were usually collected by the researchers each morning. On the day when multiple urine voids were collected, samples were delivered to the field laboratory in the afternoon and on the following morning. All urine samples were processed as previously described (Calderon et al., 1999). In the pilot study, a single FMV was collected from participants at the time of blood collection.

Venous blood samples were collected from pilot study participants using 7-ml vacutainers containing EDTA as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Blood samples for preparation of serum were collected in 7 ml vacutainers SST® with gel and clot activator (Becton Dickinson). Blood and serum samples were maintained at 4°C and shipped to North Carolina on frozen refrigerant packs.

Analytical Methods

In the first study, iAs^{III}, iAs^{V}, MAs, and DMAs in urine were separated by reversed-phase liquid chromatography, converted to their corresponding hydrides, and detected by atomic

fluorescence spectrometry (Le and Ma, 1998). In the second study, whole blood was prepared for As analysis as previously described (Hughes et al., 2000) and separation and quantitation of iAs, MAs, and DMAs in biological samples were performed by hydride generation-atomic absorption spectrophotometry (HG-AAS) (Crecelius et al., 1986). The concentrations of Se in whole blood, serum, and urine samples were determined by HG-AAS and are hereafter termed total Se (TSe) concentrations. Samples were prepared for Se analysis by wet digestion with sequential addition of nitric, sulfuric and perchloric acids (Cox, 1980). To reduce Se^{VI} to Se^{IV}, these samples were pretreated at 90ºC for 20 min with concentrated hydrochloric acid (1 : 1 v/v). Quality control for As and Se analyses used freeze-dried urine standard references material for toxic metals (SRM 2670, National Institute of Standards and Technology, Gaithersburg, MD) and the standard reference material for water (SRM 1643c). Concentrations (g/l) of creatinine (c) in urine samples were determined by the method of Archari et al. (1983).

Statistical Methods

Associations among the various arsenic metabolites in urine were measured using graphical methods, Pearson correlation, and linear regression. In some cases, data were log transformed. SAS Statistical software (SAS Institute, Cary, NC) was used for analyses.

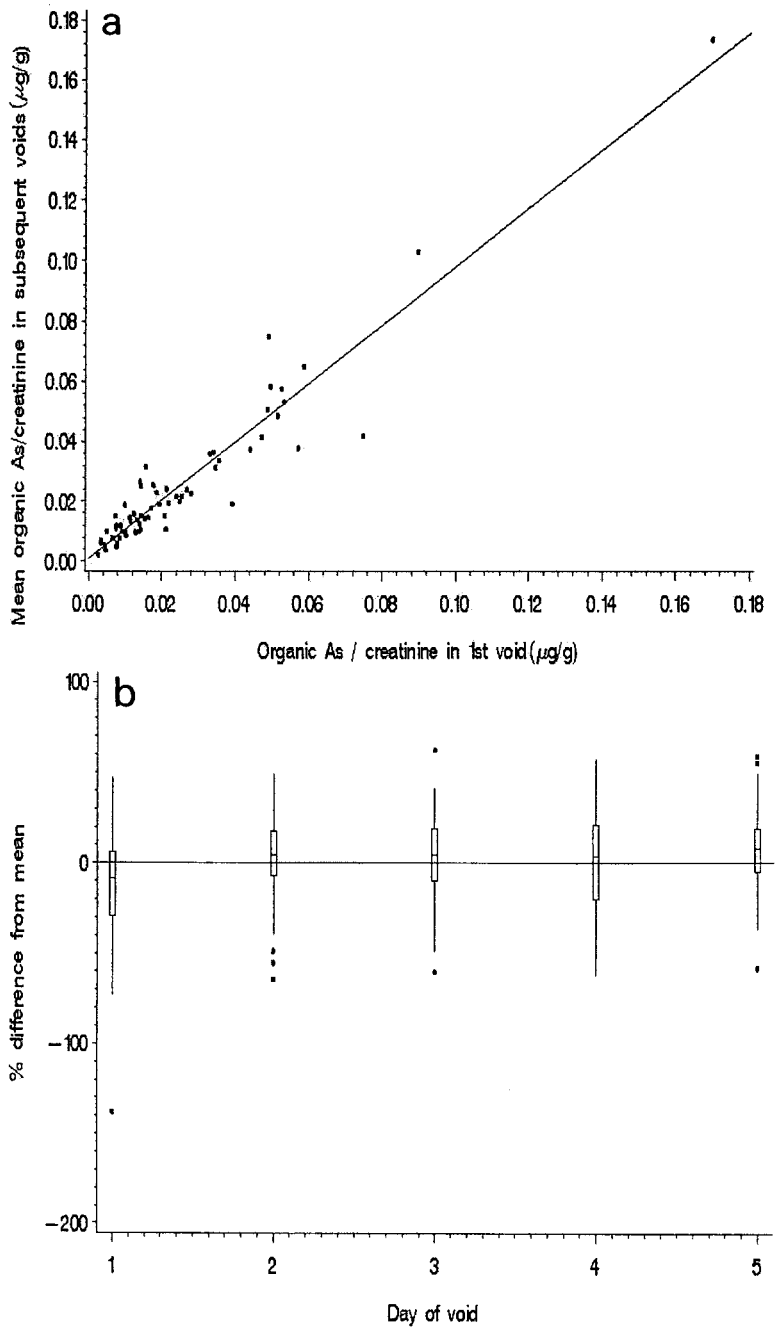
RESULTS

Study 1: Urinary Excretion of Methylated Arsenicals in a US Population Chronically Exposed to iAs in Drinking Water

This study extends the work of Calderon and associates (Calderon et al., 1999) which examined the relationship between the intensity of exposure to iAs in drinking water and the urinary excretion of all arsenical species (i.e., the sum of the concentrations of iAs, MAs, and DMAs). The present study investigated the relationship between the intensity of exposure to iAs in drinking water and the urinary excretion of the methylated species of As (i.e., the sum of the concentrations of MAs and DMAs). For these analyses, the sum of the concentrations (μg/ml) of iAs^{III}, iAs^{V}, MAs, and DMAs in urine yielded the total As (TAs) concentration and the concentrations of MAs and DMAs in urine were summed to yield an organic As (OAs) term. Thus, our approach focused on overall capacity to methylate As rather than on the production of a specific metabolite. The concentrations of iAs, MAs, DMAs, OAs, and TAs in urine were commonly expressed on a μg per g of creatinine (c) basis (e.g., iAs/c, MAs/c). The percentages of MAs and DMAs in urine were calculated by the formulae ((MAs/c / TAs/c) × 100) and ((DMAs/c / TAs/c) × 100)), respectively. The percentage OAs in urine was calculated from ((MAs/c + DMAs/c / TAs/c) × 100).

Within-day and between-day variability in the urinary output of methylated arsenicals were examined. Fig. 1a illustrates the relation between the OAs/c in the FMV and the mean of subsequent urine samples collected on the same day and between the OAs/c

Fig. 1. (a) Concordance between the concentration of organic As in the first morning void and in subsequent urine collections during a single day. Mean of organic As (sum of methyl As and dimethyl As) concentration (μg/g creatinine) in urine samples collected within 24 h, but excluding the first morning void, as a function of organic As concentration (μg/g creatinine) in the first morning void. The equation for the line is mean organic As concentration (μg/g creatinine) = 0.972 (organic As concentration (μg/g creatinine) in first morning urine sample) + 0.001. (Correlation = 0.95, $P = 0.0001$). (b). Comparison between the concentration of organic As in each first morning void and the mean concentration of organic As of the first morning voids for the 5-day study period. The distribution of the percentage differences between the concentration of organic As (μg/g creatinine) in each day's first morning void and the mean concentration of organic As (μg/g creatinine) calculated from the 5-day's first morning voids. The line inside the box is the median. The bottom and top of the box indicate the 25th and 75th percentiles. The whiskers extend ≤1.5 times the interquartile length. Observations outside the whisker range are indicated.


a
Mean organic As/creatinine in subsequent voids (μg/g)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18
Organic As / creatinine in 1st void (μg/g)
b
% difference from mean
100
0
−100
−200
1 2 3 4 5
Day of void

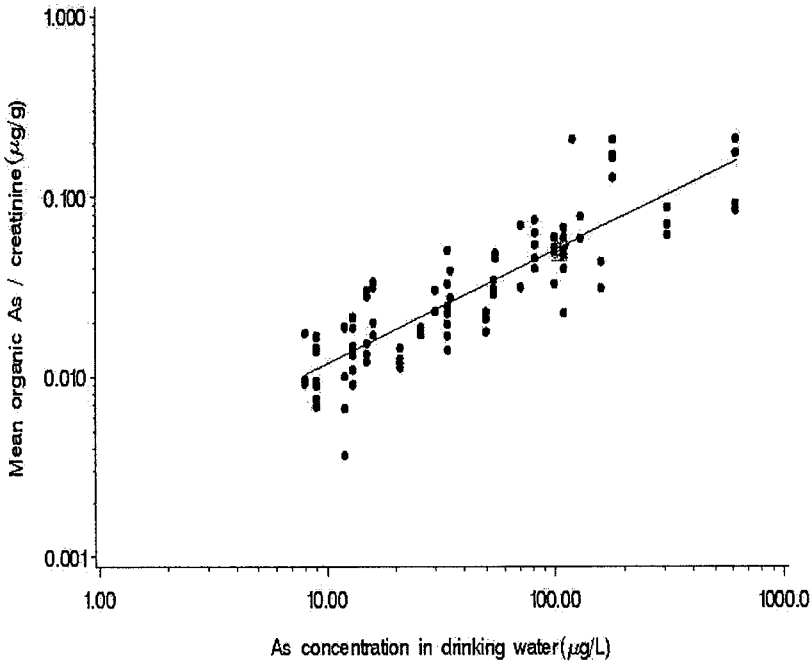
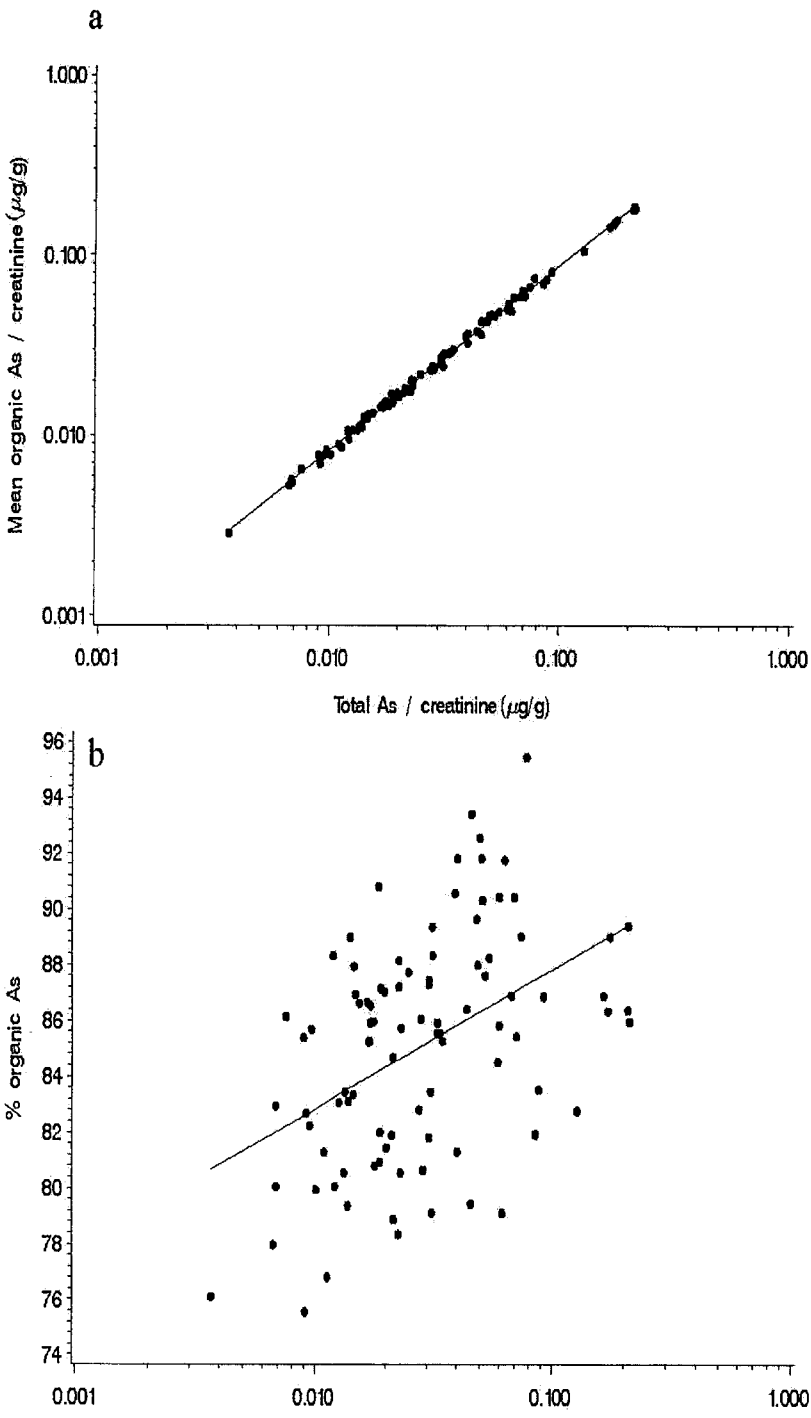


Fig. 2. Mean organic arsenic concentration (μg/g creatinine) in urine as a function of the concentration of As (μg/l) in drinking water. The equation for the line is: mean organic As concentration (μg/g creatinine) $= 10^{-2.66} \times$ (As concentration (μg/l) in drinking water)$^{0.65}$, where −2.66 and 0.65 are the intercept and slope, respectively, for the regression. (Correlation = 0.85, $P = 0.0001$).

in the FMV for each of the 5 days of the study. For within-day variability, a statistically significant correlation of 0.95 ($P = 0.0001$) was found between OAs/c in the FMV and in the mean OAs/c for the subsequent urine samples. For each participant, the OAs/c for each of the 5 FMV was used to calculate the 5-day mean FMV OAs/c. The percentage difference between each day's OAs/c in the FMV and the mean OAs/c FMV was calculated. The extent of the between-day variability in OAs/c is illustrated by a boxplot which spans the 25th to 75th percentiles (Fig. 1b). Only a few observations lie beyond these limits, suggesting OAs/c to be relatively stable over the 5-day experimental period. OAs/c was found to be a relatively stable measure of the urinary output of methylated arsenicals by study participants and subsequent analyses used the mean FMV OAs/c.

Over the concentration range of 8–620 μg of As/l in drinking water, there was a statistically significant correlation of 0.85 ($P = 0.0001$) between the concentration of iAs in drinking water and mean FMV OAs/c (Fig. 2). Because OAs accounts for most of the As in urine, a strong association (correlation = 0.99, $P = 0.0001$) was evident in the relation between the mean FMV TAs/c and the mean FMV OAs/c (Fig. 3a). A less robust but

Fig. 3. (a) Mean organic As concentration (μg/g creatinine) in urine as a function of total As concentration (μg/g creatinine) in urine. The equation for the line is: mean organic As concentration (μg/g creatinine) $= 10^{-0.030} \times$ (total As concentration (μg/g creatinine))$^{1.03}$, where −0.030 and 1.03 are the intercept and slope, respectively, for the regression of the $\log_{10}$-transformed data. (Correlation = 0.99, $P = 0.0001$). (b) Mean percentage of organic As in urine as a function of total As concentration (μg/g creatinine) in urine. (Correlation = 0.31, $P = 0.0001$)


a
1.000
0.100
0.010
0.001
Mean organic As / creatinine (μg/g)
0.001
0.010
0.100
1.000
Total As / creatinine (μg/g)
b
96
94
92
90
88
86
84
82
80
78
76
74
% organic As
0.001
0.010
0.100
1.000

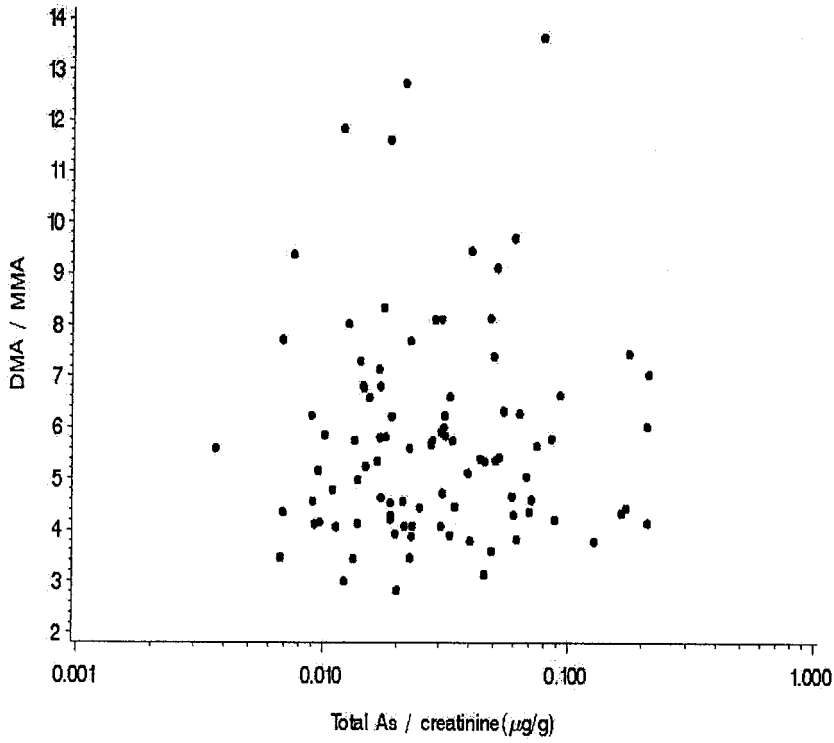


Fig. 4. The ratio of DMAs/MAs in urine as a function of total As concentration (μg/g creatinine) in urine. (Correlation = − 0.01, $P = 0.90$).

statistically significant association of 0.31 ($P = 0.0001$) was found between the mean FMV TAs/c and the mean percentage OAs (Fig. 3b).

The analysis of the urinary excretion of methylated arsenicals was extended to examine interindividual variability in the percentages of MAs and DMAs in the mean FMV. The percentage of urinary As accounted for by the summation of the percentages of MAs and DMAs ranged from 75.6 to 95.4. In the mean FMV, the percentage of MAs ranged from 6.8 to 21.4 (14.1 ± 3, mean ± SD, $n = 95$) and the percentage of DMAs ranged from 59.6 to 88.6 (71.1 ± 5.9, mean ± SD, $n = 95$). The relation between the TAs/c and the pattern and extent of methylation of As was also examined. The ratio of DMAs to MAs in the mean FMV was compared to the TAs/c in this urine sample (Fig. 4). There was substantial scatter in this relation, suggesting little correlation between the TAs/c and the amount of either MAs or DMAs in the mean FMV.

Study 2: As and Se Status of Individuals Chronically Exposed to iAs in Drinking Water

For the 16 participants from 6 families who took part in this pilot study, the concentration of iAs in drinking water ranged from 13 to 370 μg/l. The percentage of iAs^{III} in water ranged from about 7 to 50% and was not consistently associated with the concentration of iAs ($iAs^{III} + iAs^{V}$) in drinking water (data not shown). By comparison, the concentration of TSe in all water supplies used by participants was below the limit of detection (<0.8 μg/l). Table 1 summarizes data on the concentrations of TAs and TSe in blood, serum and urine. The concentration of iAs in drinking water was significantly correlated with the

TABLE 1

Concentrations of total arsenic (TAs) and total selenium (TSe) in blood, serum, and urine of individuals chronically exposed to inorganic arsenic in drinking water*

	Blood	Serum	Urine
TAs	3.3 ± 1.2 (2.3–6.3)	2.7 ± 1.1 (1.6–5.6)	39.8 ± 2.6 (10.5–96.6)
TSe	127.9 ± 26.8 (65.5–165.8)	92.4 ± 24.5 (44.2–126.3)	55.3 ± 33.2 (21.3–156.3)

* TAs is the sum of the concentrations (μg/l) of inorganic As, methyl As, and dimethyl As as determined by hydride generation-atomic absorption spectrometry. TSe is the concentration of Se (μg/l) as determined by hydride generation-atomic absorption spectrometry. Upper rows: mean ± SD, $n = 16$. Lower rows: range of values.

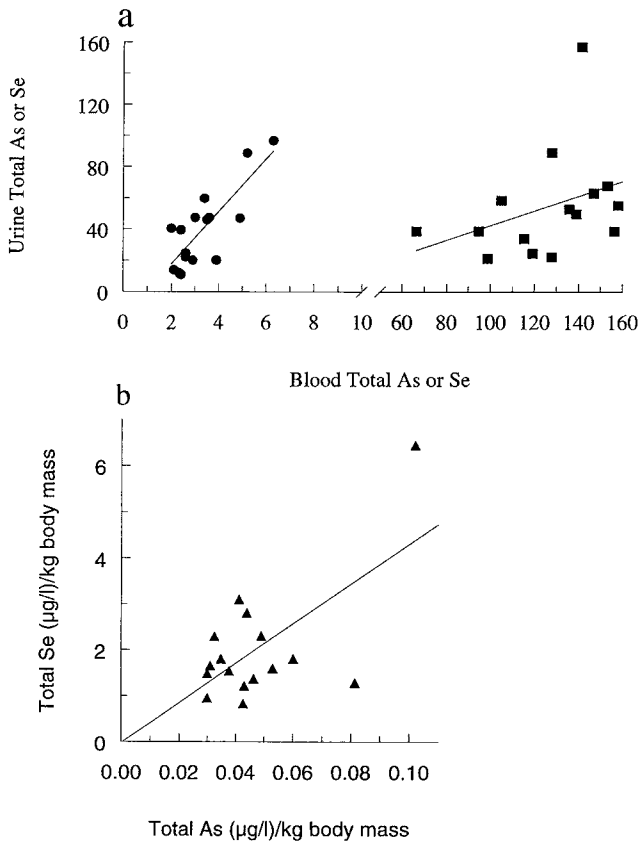


Fig. 5. (a) Relationships between the concentrations (μg/l) of arsenic or selenium in blood and urine. For arsenic (•), the equation of the regression line is $y = 16.9\ x - 16.2$, $r = 0.81$, $n = 16$. For selenium (■), the equation of the regression line is $y = 0.47\ x - 4.9$, $r = 0.38$, $n = 16$. (b). Relationship between the concentrations of arsenic and selenium in blood expressed as functions of the body weight of individuals (μg/l / kg). The equation for the regression line is $y = 43.1\ x - 0.02$, $r = 0.63$, $n = 16$.

concentration of TAs in urine ($r = 0.73$, $P < 0.0005$) and with the concentration of TAs in blood ($r = 0.51$, $P < 0.025$). There was a strong correlation ($r = 0.98$, $P < 0.0005$) between the concentrations of TAs in blood and in serum (data not shown).

Fig. 5a illustrates the associations between the concentrations of TAs or TSe in blood

and in urine. The correlation between the concentration of TAs in blood and in urine ($r = 0.81$, $P < 0.005$) was significant; however, the correlation between the concentration of TSe in blood and urine was not significant ($r = 0.38$, $P > 0.05$). Additional analyses examined the interrelations between the concentrations of these metalloids in blood and in urine. When expressed on a concentration basis (μg/l), the correlations between the concentrations of TAs and TSe in blood ($r = 0.23$) or in urine ($r = 0.14$) were not significant. Expressing the concentrations of TAs and TSe in blood on the basis of body weight of each participant (μg/l / kg) resulted in a significant correlation ($r = 0.63$, $P < 0.025$) between these variables but did not raise the correlation between the concentrations of TAs and TSe in urine to statistical significance ($r = 0.27$)

DISCUSSION

The high correlations between the OAs/c in the FMV and the mean OAs/c in urine samples collected later in the day and between the 5-day mean OAs/c and each day's FMV OAs/c demonstrated that the mean FMV was a representative indicator of the output of the methylated arsenicals in urine. Over a wide range of exposure, the concentrations of iAs in drinking water and the OAs/c in the mean FMV were strongly correlated. This is consistent with our earlier report of a high correlation between the concentration of iAs in drinking water and the TAs/c in the mean FMV (Calderon et al., 1999). The high concordance also suggests that the capacity to methylate As in individuals chronically exposed to up to about 600 μg of iAs/l in drinking water was not saturated. Studies in other populations exposed to high concentrations of iAs in drinking water suggest the capacity to produce methylated arsenicals can be saturated. Comparison of urinary metabolites in individuals chronically exposed to low (31 μg/l) or high (408 μg/l) concentrations of iAs in drinking water found higher exposure to be associated with larger proportions of iAs and MAs in urine (Del Razo et al., 1997). This suggested that chronic consumption of highly contaminated drinking water altered methylation capacity. A study in Chilean populations chronically exposed to either 15 or 600 μg of iAs/l in drinking water also found altered production of MAs and DMAs in the higher exposure group (Hopenhayn-Rich et al., 1996). In contrast, studies in workers exposed to airborne iAs have found the proportion of MAs and DMAs in urine to be unaffected by the intensity of exposure. (Smith et al., 1977; Offergelt et al., 1992; Yager et al., 1997). Studies in other species suggest that differences in amino acid, protein, lipotrope or Se nutriture (Marafante and Vahter, 1986; Vahter and Marafante, 1987; Kenyon et al., 1997, 1999) can affect excretion of methylated arsenicals. Furthermore, we found no strong correlation between the TAs/c in the mean FMV and the DMAs:MAs ratio in the mean FMV. Other factors, including phenotypic differences in the catalytic activities of As methyltransferases, may contribute to interindividual differences in the profile of As metabolites in urine. Studies in cultured primary human hepatocytes found rates of MAs and DMAs production to vary many fold among individual donors (Styblo et al., 1999).

In the pilot study, we determined the concentrations of TAs in blood, serum, and urine. The range of concentration for TAs in blood of participants (2.3-6.3 μg/l) was lower than reported values for blood TAs of other iAs-exposed individuals (Concha et al., 1998; Pi et al., 2000), likely reflecting lower exposures to iAs for pilot study participants. Serum TAs concentrations for pilot study participants ranged from 1.6 to 5.6 μg/l. For individuals with minimal exposure to iAs, serum TAs concentrations ranging from 1.5 to 5.2 μg/l have been reported (De Kimpe et al., 1993; Mayer et al., 1993; Zhang et al., 1996; Cornelis et al., 1996).

We found a statistically significant correlation between the concentration of iAs in drinking water and the concentration of TAs in blood. The linearity of the relation between the concentration of iAs in drinking water and the concentration of TAs in blood suggested that accumulation of TAs in blood was not dosage dependent. The concentrations of TAs

in blood and in urine were strongly correlated, suggesting that the concentration of TAs in urine might be a useful surrogate for the concentration of TAs in blood.

Exposure of pilot study participants to Se was assessed by determination of the concentrations of TSe in blood, serum, and urine. The concentrations of TSe in blood of participants in the current study ranged from 65.5 to 165.8 μg/l and was comparable with the level of Se found in blood (197.4 μg/l) of apparently well-nourished individuals (Whanger et al., 1988). The concentrations of TSe in serum of pilot study participants ranged from 44.2 to 126.3 μg/l and were comparable to previously reported average concentrations of TSe in serum (Pleban et al., 1982; Levander and Morris, 1984). The range of concentrations of TSe in the spot urine samples of study participants (21.3–156.3 μg/l) was comparable with that found in individuals from a US population with no untoward exposure to Se from environmental sources (34.7 μg/l) (Valentine et al., 1978). We did not find a significant correlation between the concentration of TSe in blood and in urine of pilot study participants.

We found a significant correlation between the ratios of the concentrations of TSe in urine and in blood and the ratios of the concentrations of TAs in urine and blood. We also found a significant correlation between the concentrations of TAs and TSe in blood when we expressed these variables on the basis of body mass (μmol/l/kg). Body mass has been shown in earlier studies to affect the output of TAs in urine (Buchet et al., 1996) and to confound the relationship between the intensity of exposure to iAs from drinking water and the percentage of urinary As that is present as MAs and DMAs (Thomas et al., unpublished observations). Expression of the concentrations of As in blood or urine on the basis of body mass reflects an underlying relationship between body size and capacity to retain iAs and its methylated metabolites. Consideration of body mass as an important biological variable may be warranted in future studies of dose–response relationships for arsenicals in blood and urine. Recent work has identified an As–Se complex, seleno-bis(*S*-glutathionyl) arsinium ion, in the bile of rabbits that received coadministered doses of iAs^{III} and Se^{IV} (Gailer et al., 2000) and a similar stoichiometric complex could be present in blood. However, as the concentration of TSe in blood is much higher on a molar basis than the concentrations of TAs in blood, it could account for only a small fraction of the blood Se but could be a significant component of the blood As burden. Identification of an As–Se complex in biological fluids might provide a useful biomarker of As–Se interactions.

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Arsenic Exposure and Health Effects IV
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Arsenic Methylation and the *S*-Adenosylmethionine-Mediated Transmethylation/Transsulfuration Pathway

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ABSTRACT

Studies of arsenic and its methylated metabolites in the urine of humans demonstrate that there is considerable variation in the methylation of arsenic. One of the biochemical pathways that may influence the degree of methylation is the *S*-adenosylmethionine transmethylation/transsulfuration pathway. This pathway is the site of a number of known genetic defects influencing the fate of methionine and various intermediary metabolites. Although the homozygous defects are fairly rare, little is known about the distribution of heterozygosity in different populations and its impact on transmethylation reactions. Other factors that influence the pathway include the presence of competing substrates for methyl groups such as norepinephrine, cobalamin and phosphatidyl ethanolamine. The fate of intermediary metabolites such as homocysteine, and the demand for, and dietary adequacy of the sulfur-containing amino acids (methionine and cysteine) are other determinants that influence pathway function. Insufficient levels of the B vitamins (folic acid, cobalamin and vitamin B-6) can place additional stress on methylation efficacy. Each of these variables is considered from the perspective of its ability to affect the methylation of arsenic either directly or indirectly.

Keywords: arsenic; *S*-adenosylmethionine; urinary metabolites; genetics; nutrition; transmethylation

INTRODUCTION

Arsenic can interact with several metabolic pathways in humans and influence the structure and function of biological substrates. For example, arsenate (+5) is a phosphate analog and can substitute for phosphate during the phosphorylation of ATP; the resulting compound is unstable and hydrolyzes spontaneously (Crane and Lipmann, 1953; Azzone and Ernster, 1961). In the +3 state (arsenite), it is a noted enzyme inhibitor (Webb, 1966) and can affect such processes as DNA repair (Rossman, 1998). In addition, placing experimental animals on a low arsenic diet can depress *S*-adenosylmethionine decarboxylase activity resulting in lower tissue concentrations of spermidine and spermine (Uthus, 1992). Furthermore, arsenic is methylated by humans and most animals (Aposhian et al., 1997). Thus, it may be impacted by metabolic pathways involving methylation and factors, such as nutrition and genetics, that may affect the methyl donor pool.

Studies of urinary methylated arsenic species in human populations have noted considerable variability between and among populations in the amounts of inorganic arsenic (arsenate and arsenite), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) excreted (Vahter et al., 1995; Hsu et al., 1997; Concha et al., 1998; NRC, 1999; Vahter, 1999). A number of endogenous and exogenous factors have been hypothesized to contribute to this variability.

Arsenic methylation utilizes *S*-adenosylmethionine (SAM) as a methyl donor (Aposhian et al., 1997). Methionine is an essential dietary amino acid for humans and provides the sulfur for the endogenous synthesis of the nonessential amino acid, cysteine (Stipanuk, 2000). The demands on dietary methionine for protein synthesis, transmethylation reactions and transsulfuration reactions are probably factors that influence variability in the production of methylated arsenic species. However, the interactions that affect SAM-mediated reactions are complex. They provide for the synthesis of critical intermediary metabolites, are subject to genetic heterogeneity, and have obligate requirements for several nutritional cofactors. Accordingly, there are no simple answers on the effects of nutrition, genetics and the availability of methyl donors used in the metabolism of arsenic.

Three major factors are known to influence SAM metabolism:

(1) demands for SAM by competing enzymes;
(2) essential nutritional requirements for transmethylation and transsulfuration; and
(3) genetic defects impacting transmethylation and transsulfuration reactions.

Each of these factors will be discussed in this report along with its potential contribution to the reported variability in observed human response to arsenic. The aim of this communication is to stimulate hypothesis formation concerning the potential impact of diet, genetics and growth or repair on arsenic metabolism.

S-ADENOSYLMETHIONINE

SAM, the critical reactant for transmethylation, is synthesized from methionine and ATP in a unique reaction whereby the adenosyl moiety is transferred to the sulfur of methionine liberating pyrophosphate and inorganic phosphate via the enzymatic activity of methionine adenosyltransferase, (Michal, 1999). Methionine adenosyltransferase activity is most active in the liver but occurs in other tissues as well (Ubagal et al., 1995).

Fig. 1 is a summary of various metabolic reactions linked to SAM and illustrates its diversity and importance. SAM functions to methylate an assortment of acceptor molecules and to transfer sulfur to endogenously generated sulfur-containing compounds, one of which is cysteine (Michal, 1999). If the sulfur is not diverted into the production of the sulfur-containing molecules, the demethylated methionine (homocysteine) can be remethylated, thereby, regenerating methionine. In normal circumstances, it is estimated that a single methionine that enters into transmethylation reactions can be regenerated about

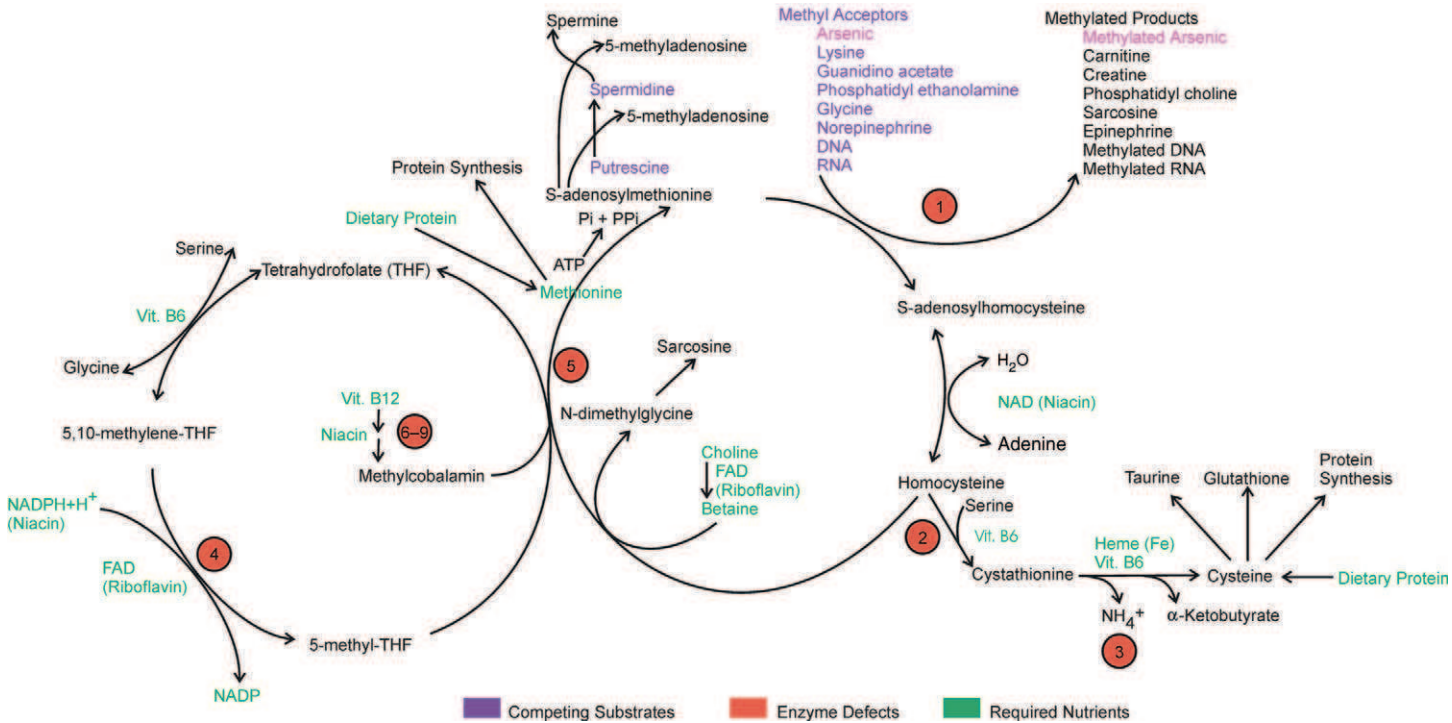

Fig. 1. *S*-Adenosylmethionine-linked metabolism. Italic type indicates competing substrates. Shaded circles indicate potential enzyme defects and shaded type indicates required nutrients. (Refer to Table 4.)

1.5–2.0 times (Mudd et al., 1989; Selhub, 1999). SAM-mediated transmethylation and transsulfuration reactions are impacted by complex feedback responses to various metabolites which will not be discussed in this paper (see Selhub, 1999).

TRANSMETHYLATION REACTIONS

Table 1 summarizes many of the physiological substrates that compete for the pool of methyl groups transferred via SAM (Michal, 1999). In addition to identifying the methylation substrate, the table also includes the name of the responsible enzyme and its methylated product. Each of the substrates listed in Table 1 is of critical metabolic importance. DNA and RNA are central to cellular function and propagation. The methylation status of these biopolymers is important to the regulation of their activity. Phosphatidyl choline is a phospholipid found in cell membranes and, thus, must be available to support cell division, and growth. Spermine and spermidine are polyamines that stabilize DNA in the chromosomes.

A second cluster of methyl group acceptors are involved with energy metabolism (Fig. 1). Trimethyllysine is used to make carnitine which transports fatty acids into mitochondria for the production of ATP and acetyl CoA via beta oxidation. Creatine can accept phosphate from ATP when it is present in excess and provides a reservoir of high energy phosphate in muscle cells to maintain activity when energy demands exceed the amount of ATP that can be provided by the glucose in muscle. This maintains muscle activity as cellular metabolism adjusts to draw on glycogen for additional ATP and mobilize glucose from the liver. Epinephrine is a hormone that enables the cell to respond to stress by increasing glycogen utilization. In addition, it modulates the flow of fatty acids to and from cells (Montgomery et al., 1990).

Two other products that are generated via SAM transmethylation are sarcosine and methylcobalamin (see Table 1). Both are noteworthy because they play a role in the methionine regeneration process. Methylcobalamin is a cofactor for the enzyme methionine synthase. Sarcosine can donate its methyl group to 5,10-methylene tetrahydrofolate via the activity of sarcosine dehydrogenase (Michal, 1999). It is also formed during the demethylation of betaine. It is unique, and illustrative of the importance of SAM reactions, that among the acceptors of its methyl group are metabolites that insure that at least a portion of the methyl groups will be recaptured. This insures availability of methionine for protein synthesis.

Metabolites which accept the methyl group from SAM function to sustain energy

TABLE 1

Competing enzymes/substrates

Enzyme	Substrate	Product
Arsenic methyltransferases	Arsenic	Monomethylarsonic acid, dimethylarsinic acid
ε-*N*-Lysine methyltransferase	Lysine (protein-bound)	Trimethyllysine (protein-bound)
Guanidinoacetate methyltransferase	Guanidino acetate	Creatine
Phosphatidyl ethanolamine methyltransferases	Phosphatidyl ethanolamine	Phosphatidyl choline
Glycine *N*-methyltransferase (liver/kidney)	Glycine	Sarcosine
Phenylethanolamine methyltransferase	Norepinephrine	Epinephrine
Methionine synthase	Cob(II)alamin	Methylcobalamin
RNA methyltransferases	RNA	Methylated RNA
DNA methyltransferases	DNA	Methylated DNA
Spermidine synthase	Putrescene	Spermidine
Spermine synthase	Spermidine	Spermine

metabolism, protein synthesis, and cell division. Since each of these functions is essential to survival of the organism, it can be hypothesized that, in situations where the amount of SAM is limiting, production of these critical cell products will be favored over methylation of less critical substrates such as arsenic. Ultimately the fate of SAM is determined by the relative availability and kinetics of the enzymes competing for this substrate. Interestingly, the production of creatine appears to be a major use of methyl groups in healthy adult males (Mudd et al., 1989).

TRANSSULFURATION

Once the methyl group has been removed from methionine leaving *S*-adenosylhomocysteine (SAH) there are two metabolic options (Michal, 1999). The first option will transfer the homocysteine sulfur to serine as an acceptor, thereby generating cystathionine and then the nonessential amino acid cysteine (Fig. 1). Transsulfuration enzymes are distributed primarily in the liver, kidney, small intestine and pancreas (Finkelstein, 1998; House et al., 1999; Selhub, 1999). The need for the transsulfuration pathway is diminished in situations where the diet provides adequate cysteine. Dietary cysteine or the cysteine generated from methionine is utilized in the synthesis of proteins, the formation of the versatile tripeptide, glutathione (which also functions in arsenic metabolism), the production of taurine, and inorganic sulfate (House et al., 1999; Kraus et al., 1998).

METHIONINE REGENERATION

Another metabolic option for homocysteine is remethylation to generate methionine. This can occur by either of two routes. The first pathway for methionine regeneration utilizes betaine as a methyl donor and functions only in the human liver and kidney (House et al., 1999). Betaine is generated by the oxidation of choline using flavine adenine dinucleotide (FAD) as the oxidizing agent (Michal, 1999). The second pathway which is available in all tissues utilizes methylcobalamin, a form of vitamin B-12 and 5-methyltetrahydrofolate to regenerate methionine (House et al., 1999; Stipanuk, 2000). As methionine becomes limiting, the regeneration of methionine is increased if the required nutritional cofactors are present. In the presence of the required cofactors, methionine can be regenerated from homocysteine for as many as four cycles (Selhub, 1999).

The flow of sulfur and carbon through the SAM-linked metabolic pathways can impact the availability of methyl groups for the methylation of exogenous substances. Arsenic methyltransferases have been identified in the liver and other tissues (Aposhian et al., 1999). As the methyltransferases transfer methyl groups from SAM to arsenic, they first form MMA and then, with transfer of a second methyl group, DMA. Based on the interaction of compounds in the SAM-linked metabolic pathways in Fig. 1, the following conditions could limit the methylation of arsenic:

- A low protein diet.
- A low methionine diet.
- A low methionine diet that is also limiting in cysteine.
- Periods of active growth that divert methionine and cysteine to protein synthesis rather than to methyltransferases.
- Deficiencies of the nutritional cofactors required for methionine regeneration.
- Periods of rapid cell division that increase the requirements for spermine and spermidine generation and polynucleotide methylation.
- Periods of active muscular activity that increase demands for carnitine and creatine.
- Metabolically demanding situations such as pregnancy and lactation.

Independent of genetic factors, one would expect that the production and excretion of methylated arsenic species as measured in plasma and urine would be lower in nutrition-

ally challenged populations, particularly those with inadequate animal protein and poor intake of the B-complex vitamins during periods of active growth and tissue repair. The severity of the problem would increase with the magnitude of the arsenic exposure.

Changes in metabolic processes could affect the concentrations of potentially toxic arsenic compounds in humans. One possibility is that there could be an increased level of the inorganic arsenic species in the body due to lowered metabolic rates. Another possibility is that during methylation of arsenic, MMA (+5) is reduced to MMA (+3) prior to the second methylation to produce DMA (+5) and the decrease in methyl donors could lead to an increased level of MMA (+3) in tissues. It was once believed that MMA (+3) was transitory in nature, but recently Aposhian et al. (2000) isolated MMA (+3) from human urine. This finding is of considerable potential importance as MMA (+3) is more toxic to some in vitro systems than arsenite (+3), the putative toxic form (Petrick et al., 2000; Styblo et al., 2000). These considerations suggest that individuals with diets low in high quality protein and B vitamins could be populations potentially sensitive to arsenic, especially during pregnancy, lactation, early childhood and adolescence. They also provide an explanation as to why populations suffering from malnutrition can have a greater biological response to arsenic than well-nourished populations. For example, lack of proper nutrition in Taiwan (Hsueh et al., 1995) and in India (Mandal et al., 1996) has been linked to increased expression of some adverse health effects after arsenic exposure.

Several investigators have observed a change in the extent of arsenic methylation with increasing arsenic exposure. Hsu et al. (1997) found that there was an increase in inorganic arsenic and MMA excretion and a decrease in the excretion of DMA as arsenic exposures increased among residents of North Taiwan. There was an accompanying increase in the MMA/DMA ratio. The subjects of this study had diets heavily reliant on rice and indigenous fish. Participants were requested to refrain from fish consumption (a source of arsenobetaine) for the 48-h period before urine samples were collected. The changes in the patterns for excretion of arsenic metabolites were most apparent at arsenic concentrations of greater than 100 μg/l.

Researchers have hypothesized that the decrease in DMA excretion observed by Hsu et al. (1997) results from enzyme saturation at the higher arsenic concentration (Vahter, 1999). However a lower affinity of MMA methyltransferase for SAM relative to physiological substrates is an alternate hypothesis for this observation. The higher Michaelis–Menton constant for the methyltransferase enzyme would conserve SAM for methyl transfer to endogenous substrates.

Other workers have also observed differences in the excretion of inorganic arsenic, MMA and DMA, even among individuals in a relatively affluent and homogeneous socioeconomic population within the United States. An evaluation of urine samples for individuals participating in the NHEXS survey found considerable diversity among individual subjects (Pellizzari, personal communication). For example, two individuals with total urinary arsenic levels of about 35 ng/ml and no detectable arsenobetaine or arsenocholine in their urine had arsenic : MMA: DMA ratios that were very different. In one person, the concentrations of inorganic arsenic and MMA, respectively, were 2.97 and 2.47 ng/ml while no DMA was detected. In the other person, most of the arsenic was excreted as DMA (8.77 ng/ml) and the ratio of inorganic arsenic to MMA was 3.34 : 3.51 ng/ml. Mass balance considerations suggest that both samples contained other arsenic-containing compounds that were not identified. Similar variability in the excretion of MMA and DMA was seen in individuals in Finland receiving the same estimated arsenic concentration from their drinking water supplies (Kurttio et al., 1998).

In Argentina, growing children excreted higher amounts of inorganic arsenic and MMA and lower amounts of DMA than adult females exposed to the same arsenic concentration in drinking water (200 μg/l; Concha et al., 1998; Vahter et al., 1995). This observation is consistent with the hypothesis that methylation capacity is diminished during periods of

active growth requiring cell division and protein synthesis. However, not all reports have found differences in arsenic metabolism in children (NRC, 1999).

A recent study of 11 males (31 ± 9 years) and 11 premenopausal females (35 ± 19 years) found significantly higher rates of transmethylation and homocysteine remethylation among the women (Fukagawa et al., 2000). This is consistent with the observations of Vahter (1999) and Hsu et al. (1997) that women seem to have a greater ability to methylate arsenic than men, strengthening the hypothesis that differences in transmethylation capacity can influence the metabolism of inorganic arsenic.

Disease is another factor that can influence arsenic methylation. For example, Geubel et al. (1988) found that patients with cirrhotic liver disease excreted more DMA than controls, but the significance of this observation is not known at the present time.

NUTRITIONAL REQUIREMENTS

It is apparent from Fig. 1 that a number of nutritional cofactors are required for transmethylation and transsulfuration reactions. As illustrated in Fig. 1, methionine, folate, cobalamin, vitamin B-6, riboflavin and niacin are critical for supplying an adequate flow of methyl groups for methylation reactions. These cofactors and their functions in the transmethylation/transsulfuration pathways are summarized in Table 2. Table 2 also includes information on the population status for each nutrient based on the most recent survey of food consumption patterns conducted by the US Department of Agriculture. The

TABLE 2

Nutritional factors

Nutrient	Function	US status (<75% of RDA) *
Calories	Provides chemical energy (ATP)	39.9%
Total protein	Provides essential amino acids and protein nitrogen for synthesis of non-essential amino acids and related compounds (e.g., purines, pyrimidines)	8.2%
Methionine	Protein synthesis, transmethylation and transsulfuration reactions; essential amino acid	No data
Methionine + cysteine	Requirements for methionine sulfur increase if cysteine is deficient in the diet since methionine sulfur is used for endogenous cysteine synthesis. cysteine is required for synthesis of endogenous proteins, glutathione, and taurine	No data
Vitamin B-6	Cofactor for cystathionine β-synthetase, γ-cystathionase, and serine hydroxymethyl-transferase	30.5%
Vitamin B-12	Methylcobalamin is a cofactor for methionine synthase	9.8%
Folic acid	Methylene tetrahydrofolate provides the methyl group for synthesis of methionine from homocysteine	18.9%
Niacin	Cofactor for methylation of cobalamin, tetrahydrofolate reductase, and *S*-adenosylhomocysteine hydrolase	10.9%
Riboflavin	A cofactor for 5,10-methylene tetrahydrofolate reductase	13.6%
Choline	Oxidized to betaine, a source of methyl groups for methionine synthase in the liver	No data
Iron	Heme is a cofactor for γ-cystathionase activity	21.2%

* Data from USDA (1998).

percentage given represents the percentage of the population that routinely receives less than 75% of the Recommended Dietary Allowance (RDA) (USDA, 1998).

METHIONINE AND CYSTEINE

Methionine is essential to humans because it cannot be endogenously synthesized. Because methionine is essential, it must be supplied in the human diet on a daily basis (Stipanuk, 2000). Cysteine, on the other hand can be synthesized using the sulfur from methionine and is not essential.

There have been several attempts to estimate the dietary requirements for methionine (Table 3) and each estimate has methodological limitations. The values for adults by Rose (1957) and Leverton et al. (1959) were based on early studies of essential amino acids and are derived from balance study data. Estimates were provided for methionine and combined methionine plus cysteine. The World Health Organization values (WHO, 1985) are more recent and give a single value for methionine plus cysteine. The National Research Council (NRC, 1989) adopted the WHO recommendations for their 1989 dietary guidelines.

In the absence of adequate dietary cysteine, methionine must also provide the sulfur for cysteine synthesis. The presence of an adequate level of cysteine in the diet will reduce or spare about 30% of the requirement for methionine (NRC, 1989). However, cysteine cannot completely replace methionine and about 60% of the total requirement for methionine/cysteine must be provided as methionine in the diet.

The methionine plus cysteine requirements for infants and children on a mg/kg body weight basis are higher than those for adults. The neonate needs about 58 mg/kg/day, the requirement for a 2-year-old is about 27 mg/kg/day and that for a 10-year-old about is about 22 mg/kg/day. The value for adults is considerably lower at about 13 mg/kg/day (NRC, 1989).

Diets that are low in protein or are constituted from poor quality protein may not provide adequate methionine to supply the metabolic demand for protein synthesis as well as that for methylation of intermediary metabolites, biopolymers such as DNA and RNA, and exogenous materials (Table 2). When methionine is deficient, these metabolic functions are compromised. The nature of the metabolic adjustment will be complex, impacted by enzyme kinetics, tissue localization of enzymes, physiological status (i.e., growth, tissue repair, pregnancy, lactation) and nutritional status. Methionine is the limiting amino acid in soy protein, lentils, chick peas and some bean species (Jansen, 1981). Populations reliant on these foods for much of their protein requirement may be more vulnerable to arsenic exposure.

A state of methyl group stress may exist in populations where protein quality and quantity are deficient. Some of the populations that have displayed the greatest frequency of arsenic-linked health effects (Taiwan, Bangladesh, Bengal) are among those that can be characterized as nutritionally vulnerable populations. Even among well-nourished coun-

TABLE 3

Estimates of dietary methionine requirements [*]

Estimate	Methionine (mg/day)	Methionine + cysteine (mg/day)
Rose, 1957 (men)	165	1100
Leverton et al., 1959 (adjusted, women)	287	1827
WHO, 1985 (adults)	–	845

[*] Adapted from Stipanuk, 2000.

tries, such as the US, there are segments of the population that receive less than the recommended level of dietary protein and, thus, might be more likely to respond adversely to arsenic exposure. As indicated in Table 2, the 1994–1996 Continuing Survey of Food Consumption conducted by the US Department of Agriculture found that about 8% of the population received less than 75% of the recommended dietary allowance (RDA) for protein (USDA, 1998). Protein deficiencies most often accompany deficiencies in calories and other nutrients and are seen among children more frequently than adults (NRC, 1989).

VITAMIN COFACTORS

Folate, cobalamin, vitamin B-6, riboflavin and niacin are required for the regeneration of methionine in extrahepatic tissues. Niacin is necessary to convert adenosylcobalamin to methylcobalamin and 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate. Paradoxically, one of the pathways for the conversion of adenosylcobalamin to methylcobalamin uses SAM as the methyl donor (Michal, 1999; Stipanuk, 2000). Folate and cobalamin are frequently deficient in women of childbearing age and the elderly. The USDA (1998) survey found that nearly 20% of the population received less than 75% of the 1989 RDA for folate (USDA, 1998). About 10% of the population had diets containing less than 75% of the RDA for vitamin B-12 and niacin (USDA, 1998).

Folate fortification of grains was initiated in 1998 and the RDAs for folate were increased from their 1989 values (IOM, 1998). Accordingly, the folate status of the populations requires reevaluation. However, folate is still considered to be a problem nutrient for Americans, especially among pregnant women (IOM, 1998).

Choline is important because it functions in the hepatic remethylation of methionine. Dietary deficiencies of choline are likely to occur only in times of active tissue regeneration or growth where endogenous synthesis cannot supply adequate levels to sustain cell growth and division (IOM, 1998).

The remaining nutrients in Table 2 function to insure adequate levels of cysteine in situations where cysteine is limited in the diet and are less likely to be perturbed by arsenic exposure unless there is a simultaneous deficiency in total protein. However, any decrease in the production of glutathione secondary to a deficiency in cysteine could impact the toxicity of inorganic arsenic via a different mechanism as GSH is required for the reduction of MMA (+5) to MMA (+3) (Thompson, 1993).

There are some experimental data that illustrate the importance of adequate nutrition to arsenic metabolism. Vahter and Marafante (1987) have demonstrated that diets low in methionine, choline or protein reduce arsenic methylation in animals and increase its tissue binding. Although there are few human data on the impact of the remethylation nutrients on arsenic metabolism, there are human studies that demonstrate that low folate, vitamin B12 and vitamin B6 status increase serum homocysteine levels (Cravo et al., 1996; IOM, 1998; Wilcken and Wilcken, 1998). Elevated homocysteine, when not due to one of the genetic factors discussed below signifies either decreased transsulfuration of homocysteine or decreased remethylation in extra hepatic tissues.

GENETIC VARIABILITY

The metabolic complexities of transmethylation and transsulfuration provide an explanation for why the response of a poorly nourished populations to arsenic may differ from that of a well nourished population. However, the fundamental metabolic considerations discussed above are made even more complex by genetic variations in the enzymes responsible for these reactions. Nine of the enzymes in Fig. 1 have one or more genetic defects which are found among humans. The homozygous defects that have been identified are summarized in Table 4.

TABLE 4

Genetic factors and enzyme defects

Enzyme number*	Defect	Consequences	Comments
1	Methionine adenosyltransferase deficiency	Hypermethioninemia	• Autosomal recessive • Some tested heterozygotes have displayed reduced enzyme activity
2	Cystathionine B synthetase deficiency	Hypermethioninemia, hyperhomocysteninuria, cystathionuria	• Autosomal recessive • Most common transsulfuration disorder • Heterozygotes tend to have less than 50% of normal enzyme activity
3	γ-Cystathionase deficiency	Cystathionuria	• Autosomal recessive
4	Methylene tetrahydrofolate reductase deficiency	Hyperhomocysteinemia, cystathionuria	• Autosomal recessive • Decreased enzyme activity in heterozygotes • Several genetic variants
5	Methionine synthase deficiency	Hyperhomocystenemia, cystathionuria	• Autosomal recessive
6, 7	Cobalamin C and D disorders	Hyperhomocystenemia, cystathionuria	• Autosomal recessive
8, 9	Cobalamin E and G disorders	Hyperhomocystenemia, cystathionuria	• Autosomal recessive

* Numbers refer to Fig. 1.

A methionine adenosyltransferase deficiency appears to be relatively rare. In this situation, SAM production is impaired and levels of methionine in serum and urine are elevated (Ubagal et al., 1995). At least three variant enzyme isoforms have been identified (Finkelstein, 1998; Ubagal et al., 1995). The α- and β-forms predominate in the adult liver and appear to be the product of a single gene. The γ-form has been identified in fetal liver, kidney, brains, lymphocytes, and other peripheral tissues and seems to originate from a second gene. The disorder does not generally present with clinical signs which limits determining its prevalence in the population. However, some affected individuals present with neurological problems and learning defects (Ubagal et al., 1995). The presence of this defect (homozygous or heterozygous) in individuals would further aggravate the influence of the dietary factors discussed above on the cellular metabolism of arsenic.

Identified defects in cystathionine β-synthetase, γ-cystathionase, 5,10-methylene tetrahydrofolate reductase (MTHFR), and methionine synthase (Mudd et al., 1989; Rosenblatt, 1989) along with cobalamin disorders (Fenton and Rosenberg, 1989; Rosenblatt, 1989) all lead to elevated homocysteine levels. Cystathionine levels in plasma and urine are also increased for all but the γ-cystathionase deficiency. The defects in cystathionine β-synthetase and γ-cystathionase affect transsulfuration while the cobalamin and MTHFR defects alter homocysteine methylation reactions.

Several studies suggest a link between elevated homocysteine levels and cardiovascular disease in humans (Cravo et al., 1996; Wilcken and Wilcken, 1998; de Jong et al., 1999). Data on homocysteine and cystathionine in arsenic exposed populations might be helpful in evaluating the risk of cardiovascular effects from arsenic exposures (Chen et al., 1996; Lewis et al., 1999).

There is some clinical evidence that a defect of MTHFR may increase the toxicity of

arsenic. A 16-year-old girl in a family of seven was the only member to exhibit neurotoxicity from exposure to an arsenical pesticide and she was also the only family member to suffer from a MTHFR deficiency. Lowered MTHFR activity would depress homocysteine methylation and the resulting deficiency in methyl groups could provide an explanation for her sensitivity (Brouwer et al., 1992).

The distribution of cobalamin disorders (C, D, E, and G) in the population is not known. Carriers of the trait are not symptomatic but do have a compromised ability to support remethylation of homocysteine to form methionine. The problem for the C and D disorders is a defect in the synthesis of methylcobalamin from precursor vitamin B-12 forms (Linnell and Bhatt, 1995). The defect for the E and G disorders is a defect in the ability of methionine synthase to utilize methylcobalamin (Linnell and Bhatt, 1995). There may be an additional defect in the structure of methionine synthase apart from the E and G cobalamin disorders.

CONCLUSIONS

Consideration of metabolic demands on the transmethylation and transsulfuration pathways leads to several hypotheses regarding factors that could impact arsenic methylation and thereby influence the toxicity of ingested inorganic arsenic. These factors would not impact the toxicity of arsenobetaine, arsenocholine, or dimethylarsinic acid from the diet but could influence the metabolism of inorganic arsenite and arsenate and both valence states (+3 or +5) of MMA. These hypotheses can be summarized as follows:

- Diets deficient in total protein, animal protein, and B vitamins may decrease arsenic methylation.
- Arsenic methylation may be decreased in periods of active cell division and growth unless there is excess methionine in the diet.
- Genetic defects in methylcobalamin synthesis and MTHFR will decrease homocysteine remethylation in individuals with marginal protein diets and possibly arsenic methylation. Increased serum homocysteine may be a biomarker for this condition.
- Studies of urinary arsenic metabolites among individuals with elevated serum homocysteine or cystathionine may provide insight to the role of methyl group availability in arsenic metabolism.
- Within certain segments of the US population, there may exist nutritional deficiencies which may impair transmethylation and transsulfuration reactions and thereby increase the concentrations of inorganic arsenic in the tissues of exposed individuals.
- Heterozygosity in transmethylation/transsulfuration enzymes leads to reduced efficiency in both pathways and thereby affects the pool of methyl groups available for arsenic methylation.

Understanding the mode of action for the carcinogenic and noncancer effects of arsenic in humans has been difficult. The problem is due in part to the lack of a good animal model and confounded by the diversity in responses seen among individuals exposed to inorganic arsenic through the diet and drinking water. Through this paper, the authors hope to stimulate consideration of the physiological, nutritional, and genetic factors that can influence arsenic methylation in humans as new studies of arsenic are planned and conducted. Since there are many factors that can influence arsenic methylation, we believe that it is essential to examine a variety of biological factors rather than measuring a single variable and assuming that nutrition and methyltransferase activity is/is not adequate. The aim of this communication is to demonstrate that alterations in the ability of individuals to methylate arsenic may be influenced by a number of factors other than just the activity of the arsenic methyltransferases.

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An Overview of the UNICEF Supported Arsenic Mitigation Programme in Bangladesh

C.J. Davis

ABSTRACT

UNICEF is supporting Governments' response to the problem of arsenic contamination in groundwater in Bangladesh, India and elsewhere through a series of comprehensive mitigation programmes. At the country level, UNICEF works directly with Government bodies such as the Public Health Engineering departments in India and Bangladesh. UNICEF also implements joint programmes in cooperation with Governments at both national and sub-national levels with NGOs such as BRAC, Grameen Bank and DCH in Bangladesh. Globally UNICEF works closely with partner organizations including WHO and others to coordinate responses and disseminate information.

UNICEF-supported arsenic mitigation programmes focus on four elements: (1) arsenic testing: the use of field and laboratory techniques for the blanket testing of public and private water points in arsenic-suspect areas; (2) alternative water sources: support for the provision of arsenic-free water using a variety of technologies and methodologies, from arsenic-removal to rain water harvesting; (3) communication campaigns: design and delivery of comprehensive messages in a variety of media, from the national mass media to interpersonal levels; (4) health-related interventions: including patient identification, health worker training and support to provide basic care.

This presentation provides an overview of these programme components with implementation examples primarily from Bangladesh and India.

Keywords: UNICEF; Bangladesh; tubewells; arsenicosis; DPHE-UNICEF-NGO5 Thana Project

INTRODUCTION

UNICEF first became involved in water supply in Bangladesh, when assistance was given to repair approximately 10,000 wells following war and a devastating cyclone in the early 1970s. UNICEF continued to provide support thereafter, working with the Department of Public Health Engineering (DPHE) in the Ministry of Local Government and Rural Development Co-operatives.

Following reports in the mid 1990s that there may be arsenic contamination in ground water, in 1996 UNICEF funded a large scale tube well testing programme which was implemented by the DPHE, UNICEF's traditional counterpart for water supply and sanitation in Bangladesh. A total of 51,000 tube wells were tested, this was one of the earliest and at the time, most comprehensive national surveys (from a national total of anywhere between 5 and 10 million tube wells, two-thirds of which are estimated to be privately owned). Results indicated that there was indeed widespread contamination ranging from the north to the west and the east. The Bangladesh government has set the safe level of arsenic contamination at 0.05 mg/l. The testing programme indicated that 29% of those wells tested are contaminated above a level of 0.05 mg/l, affecting 211 Thanas (sub-districts) out of a total of 460 Thanas. Subsequently, a study funded by DFID (UK) and carried out by the British Geological Survey (BGS) in conjunction with the Government of Bangladesh Ministry of Local Government and Co-operatives and the Department of Public Health Engineering and presented in Dhaka on the 30th March 2000, suggests that contamination is more widespread and possibly covers 247 Thanas.

While the number of arsenicosis patients so far identified is between 7500 and 8000, it is generally thought that over 22 million people are potentially drinking water at arsenic concentration levels greater than 0.05 mg/l. The risk of drinking such water is arsenicosis. Patients may go on to develop gangrene and cancer, conditions which result in disability and death. Little is understood even today of the epidemiology of arsenicosis and many questions remain unanswered, such as why some members of a family develop the skin nodules and other signs of arsenicosis, while others do not, even though they are consuming water from the same source.

Arsenic is found in groundwater at between 10 and 50 m depth. Wells as deep as 150 m may also contain arsenic, but depths beyond 200 m are suggested (by the BGS report) to be safe, although, again, recent reports propose that even at these greater depths there is no surety of safety. Until a more comprehensive national survey is completed, the exact situation will not be known.

It is indicative of how little was known about the possibility of arsenic contamination in ground water that there were, at the time of implementing the first test programme, few choices available with regard to field-test kits. The one used was the MERCK kit, which had the limitation of being unable to measure accurately below 0.1 mg/l. Laboratory validation has been used as a cross-check of field test results.

Another concern is the extent of contamination in the food chain, due to there being widespread use of tube wells for irrigation purposes. In addition, while there are now a number of studies which are underway, the extent of contamination (if any) of the top and sub soil is not yet known.

METHODOLOGY

Clearly UNICEF, as one of the major actors in water supply and sanitation in Bangladesh since the 1970s, and together with other partners in the sector, had to be involved in efforts to mitigate the results of the arsenic problem. More and more, it was realised that the issue was becoming a race against time for many people.

While much was not known about how to deal with the arsenic contamination, avail-

able evidence indicated that if people begin to drink safe uncontaminated water then the effects of arsenicosis, if caught at an early stage, can be halted and even reversed. Project activities would logically centre on identifying those sources, which were unsafe and then help to make safe sources available.

UNICEF agreed with Government that it would develop and implement an action research project in four Thanas in the most affected areas, which began in July 1999. These would be Jhikorgacha, Bera, Kochua and Sonargaon. UNICEF's efforts are part of a broader national effort. The Government of Bangladesh has taken a World Bank loan to establish the Bangladesh Arsenic Mitigation Water Supply Programme (BAMWSP) (with World Bank and SDC funds) and other partners such as DANIDA, Care/SDC and Water Aid etc. are developing and implementing projects.

Clearly, there were a number of issues that would need to be addressed simultaneously in order to not only provide relief to the affected people but to also better understand the problem so that long-term measures could be implemented. These issues were identified as being:

(1) Blanket testing to identify not only contaminated wells, but also the non-contaminated sources.
(2) Communication to inform the people of the problem of contamination, the consequences and how to deal with, without causing panic.
(3) Patient identification and treatment.
(4) Identifying and implementing alternate safe water options.

UNICEF felt from the outset that speed was going to be of the essence. It was decided that the best way to put the project in motion as quickly as possible was to develop and implement the strategy jointly with some of the major NGOs in Bangladesh, thereby utilising their existing and extensive field and management structures. The project is a DPHE–UNICEF Action Research project, placed under the DPHE's Research and Development wing which was the quickest way to set the project up. The NGOs that were willing to participate were BRAC, Grameen Bank and the Dhaka Community Hospital (DCH, which has played a major and important role from the outset of the identification of the problem). Later on, a fifth Thana was added, with a project developed jointly with the Rotary International and Integrated Service for the Development of Children and Mothers (ISDCM) in Manakganj.

An integrated approach has been adopted. All tube wells have been tested for arsenic contamination above the acceptable limits and those, which failed painted red following government policy. Those that were tested and estimated by field test kit as being safe were painted green. People are informed not to drink or cook with the water from tube wells painted red through a mass communication campaign, which has been developed with other actors and has been cleared and approved by the Technical Advisory Group (TAG) of the BAMWSP programme and the Ministry of Health.

Arsenicosis patients were identified as quickly as was possible and palliative ointment treatment provided, along with vitamins. In this activity, the DCH led the way and trained many health workers in how to recognise arsenicosis, as well as advising all Project NGOs, and DPHE–UNICEF with regard to the medical aspects of the problem.

Lastly and importantly, several water supply options have been identified and communities helped to implement the options. These range from the simple indigenous 3-Kolshi (or clay pot) filter which had been developed in Bangladesh many years ago as a simple device to provide safe water with regard to contaminated surface sources. This device also works to remove arsenic from water providing that iron is present, which is provided by adding iron filings to the first pot. Pond water is treated and made potable through a filter system. Deep tube wells are an option. Rainwater harvesting has also been used. Local ceramic household filters are being developed by national entrepreneurs, but with limited success so far.

Efforts to develop more accurate and locally manufactured field test kits have been encouraged. Local manufacturing has proven to be more difficult than might have been expected, with a major hurdle being the quality of reagents and the ability to manufacture large quantities. Another development is an electronic device is being developed by an Austrian University Professor, which will be capable of taking a *reading* of the level of arsenic present in the test water, to 0.01 mg/l. This would be superior to the other field kits where the level is *estimated* by comparing a stain made by arsine gas on mercury bromide impregnated paper, against a colour chart. The electronic device also needs to produce arsine gas in the same way as for the other kits, but thereafter it reads rather than estimates, which is a clear advantage. While this is still in the process of being proven and certified, it may well eventually augment laboratory tests.

DPHE–UNICEF efforts for arsenic mitigation have therefore centered on an integrated approach, implemented quickly through the network and management capability of large NGOs, as an action research project; the output of which would feed into later project development and implementation for the long term.

RESULTS

In addition to the initial 51,000 tube well national survey, a total of 98,462 tube wells have been tested in the five Thana action research project to date. Out of these, 59% (58,329) have been declared as contaminated and 41% (40,133) have been declared as being safe. These results are currently being processed to be entered into and included in the Government's National Arsenic Mitigation Information Centre (NAMIC) database.

The total population in the project areas is estimated at 1,237,066. The number of arsenicosis patients so far identified in the project area is 1050. A total of 18,000 tubes of ointment have been provided together with 580,000 vitamin tablets. Sensitisation and communication meetings have been conducted at community level which total 412 in number. The number of health workers, community workers, project personnel and local masons who have been trained so far is 550. The number of alternate safe water options so far provided is 4309.

As the project has progressed, everyone involved has become aware of the terrible human dimension of this problem for the affected populations. The appearance of skin lesions, nodules and 'rain drop' pattern skin conditions is leading to those affected being ostracized from village life. Children have been asked to leave their class, girls and boys are finding it difficult to find marriage partners, husbands have left their family; all for fear that the condition may be contagious.

When people have been affected by gangrene and have had to endure surgical removal of limbs or parts of limbs, this has affected the amount of work they can do and subsequent money they can earn either through daily labour or through work in their fields. In some cases, several members of the family have been so affected, thereby seriously reducing their ability to earn sufficient funds for normal sustenance.

DISCUSSION

The project has been successful in developing and implementing a mitigation strategy in a relatively short time. Much has been learned about the problem and how to deal with it, which will help to improve future project design as activities are scaled up.

While the project experiences have been mostly positive, they also raise a number of questions for discussion. These are:

(1) How to ensure that the communication campaign reaches all people and has the desired impact, as quickly as possible?
(2) The involvement of large NGOs with their established infrastructure enabled fast

project implementation. How to maintain this momentum and at the same time ensure local government are fully involved so as to ensure sustained efforts?

(3) How can the private sector be encouraged to participate in the programme (consider that over two-thirds of all tube wells in the country are estimated to have been installed by the private sector) and how to ensure a commitment for integrity from this sector?

(4) How to co-ordinate the many studies which are being carried out by numerous agencies, in order to ensure that the results all feed into the national objective without duplication of cost and effort?

(5) How can the identification and management of people with arsenicosis be handled considering that a higher level of sophistication is called for than was originally envisaged?

(6) Field testing of water sources remains a major challenge, how to obtain more accurate field test results?

(7) How can alternate water sources be provided to the large number of affected peoples, in a sustainable way, given the large capital cost involved and the need to provide these alternate sources as quickly as possible?

CONCLUSIONS

There are enough safe water options already identified in Bangladesh so that no one really has to consume arsenic-contaminated water. The problem is how to ensure these options are appropriate and affordable at community level and implemented in a sustainable way as quickly as possible.

The DPHE–UNICEF–NGO 5 Thana project has been successful in moving quickly with an integrated approach, which is now being scaled up to include an additional 15 Thanas. This momentum needs to be sustained in the long term. For as long as there is a Bangladesh, the issue of arsenic contamination is going to exist and so it is crucially important that systems for handling the problem are established within local government and communities as soon as possible. Continued international support in terms of technical and financial resources will be important.

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Arsenic Exposure and Health Effects IV
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Seventeen Years Experience of Arsenicosis in West Bengal, India

Kshitish Chandra Saha, Dipankar Chakraborti

ABSTRACT

Exposure periods from 6 months to 10 years (average 2 years) are required to develop arsenicosis symptoms, depending on the concentration of arsenic in water, the amount of daily water intake and the nutritional status of the person consuming such water. Melanosis is the earliest sign. Melano-keratosis appearing in adults is almost a sure sign of arsenicosis. The clinical spectrum has been classified into 4 stages, 7 grades and 20 subgrades. The stages are (I) preclinical, (II) clinical, (III) complications and (IV) malignancy. In the preclinical stage (grade 0), urine, nails, hair and skin scales show high concentrations of arsenic but melanosis and keratosis are absent. The clinical stage has four grades: (1) melanosis; (2) spotted keratosis in palms and soles; (3) diffuse keratosis in palms and soles; and (4) dorsal keratosis. The complication stage (grade 5) involves lungs, liver and other organs. The malignancy stage (grade 6) develops 10–20 years after the onset of symptoms. Chelating agents are of little benefit in keratosis and the advanced stages. Malnutrition often hastens the appearance of signs, and good nutrition often has a protective influence and it delays the appearance of signs. The only way at present to prevent the progress of the disease is to stop consuming arsenic contaminated water.

Keywords: arsenical dermatosis; stages and grades of arsenicosis; clinical features of arsenicosis; clinical diagnosis of arsenicosis, malignancy

INTRODUCTION

Arsenicosis in West Bengal was first detected by K.C. Saha from the Dermatology Out patients Department of the Calcutta School of Tropical Medicine in 1982. K.C. Saha also first detected arsenicosis in Bangladesh in 1984 in the Khulna district.

During 1983 five arsenic affected villages from five blocks of four districts in West Bengal became known. The present situation (up to May 2001) is 2600 villages from 74 blocks in 9 districts out of a total of 18 where groundwater contains arsenic above 50 μg/l. Total area and population of West Bengal is 89,193 km^2 and 68 million respectively. About 42.7 million people in 9 arsenic affected districts are at risk. Extrapolation of available results indicates (Chowdhury et al., 2000) that about 6 million people are drinking arsenic contaminated water with arsenic concentration above 50 μg/l in these 9 districts and about 300,000 people are suffering from arsenical skin lesions.

MATERIALS AND METHODS

A total of 4865 patients with arsenical dermatosis (ASD) were thoroughly examined for clinical features in 300 rural areas. The field survey was initiated in 1983. It included clinical investigation, collection of tube well water samples, nails, hairs and skin scales of patients. The cases of mild complications, such as asthmatic bronchitis, received treatment in the field. However, serious cases with complications, such as ascetic jaundice, were admitted to the hospital of the Calcutta School of Tropical Medicine (CSTM), India, between 1983 and 1987 (Saha, 1984; Saha, 1995). After 1994 a collaborative study was done with the School of Environmental Studies (SOES) of Jadavpur University. In this institution arsenic content has been measured by flow injection hydride generation, atomic absorption spectrometry (FIHGAAS) (Samanta et al., 1994). Even after working for 17 years, we feel we have only seen the tip of the ice-berg.

TABLE 1

Arsenic affected blocks in West Bengal (up to 1998)

Districts	Blocks/wards
N-24 Parganas	Barasat-1, Barasat-2, Bongaon, Hashnabad, Gaighata, Deganga, Basirhat-1, Basirhat-2, Swarupnagar, Habra-1, Habra-2, Baduria, Barrackpur-1, Barrackpur-2, Haroa, Bagdah, Rajarhat, Amdanga, Hingalganj
S-24 Parganas	Baruipur, Sonarpur, Bhangar-1, Bhangar-2, Budge Budge-2, Joynagar-1, Mograhat-2, Bishnupur-1, Bishnupur-2, Canning-1
Nadia	Karimpur-1, Karimpur-2, Tehatta-1, Tehatta-2, Kaliganj, Nabadwip, Shantipur, Chakdaha, Haringhata, Ranaghat-1, Ranaghat-2, Hanskhali, Krishnaganj, Nakashipara, Chapra
Murshidabad	Farakka, Suti-1, Suti-2, Bhagabangola-2, Raghunathganj-2, Murshidabad, Domkal, Jalangi, Beldanga-1, Hariharpara, Raninagar, Naoda, Behrampur
Malda	Kaliachak-1, Kaliachak-2, Kaliachak-3, Manikchak, Englishbazar
Bardhaman	Purbasthali-1, Purbasthali-2
Haora	Shympur, Uluberia, Bali-Jagachha
Hugli	Balagarh
Calcutta	Ward number-93, 94, 95, 98, 112, 113

RESULTS AND DISCUSSIONS

West Bengal has an area of 89,193 km^2, with a population of 68 million in 18 districts. Out of these, arsenic-affected districts cover 38,865 km^2, with 42.7 million people. The affected districts are: North 24 Parganas, South 24 Parganas, Nadia, Murshidabad, Bardhaman, Haora, Hugli, Malda and southern part of Calcutta. Out of 341 blocks of West Bengal and 167 in arsenic-affected 9 districts, 69 blocks are affected (Table 1).

Symptomatology (Table 2)

In total, 4865 cases have been analysed. The phases of arsenicosis have been classified into four stages: (I) preclinical; (II) clinical; (III) complications; and (IV) malignancy. The onset is slow and insidious. Preclinical or presymptomatic phase lasts for 6 months to 10 years (average 2 years) depending on the concentration of arsenic in ground water, the amount of daily water intake and nutritional status of the person. If the arsenic concentration in consumed water is higher than 1000 μg/l, daily intake of arsenic contaminated water is more than 2 litre and malnutrition is present, then signs may start between 6 months and 2 years. If arsenic concentration is below 100 μg/l, daily water intake is about 2 litres and the consumer has a good diet, the signs may not even appear or may take very long period.

TABLE 2

Analysis of clinical features of arsenicosis (1983–1999)

	No. of cases	Percent (%)
I. Dermatological (external or cutaneous signs)		
Major dermatological signs		
Diffuse melanosis	4,865	100
Spotted melanosis (rain drop pigmentation)	2,792	57.40
Leucomelanosis	1,542	31.70
Palmo-planter keratosis	2,987	61.40
Dorsal keratosis	1,467	30.00
Minor dermatological signs		
Mucous membrane pigmentation	345	7.10
Non-pitting oedema	248	5.10
Conjunctival congestion	194	4.00
II. Non-dermatological (internal or systemic)		
Major systemic signs		
Lungs: asthmatic bronchitis	1,576	32.40
Liver: hepatomegaly	908	18.70
Spleen: splenomegaly	72	1.5
Liver fibrosis (NCPF): ascites	24	0.50
Minor systemic signs		
Weakness/anaemia	698	14.35
Burning body myalgia	82	1.68
Laryngitis–hoarseness	14	0.30
Hypothyroidism	14	0.30
Suprarenal deficiency	4	0.10
Myopathy	1	0.02
Ischaemic gangrene (black foot disease)	1	0.02
III. Malignancy		
Skin malignancy	212	4.35
Internal malignancy	38	0.78

Total number of arsenical dermatosis (ASD) cases: 4865.

Major Dermatological Signs (Stage II)

- *Melanosis:* this is the first sign and is present in 100% of cases. Diffuse melanosis appears in palms and soles (Fig. 1) and the whole body is darkened gradually, both covered and exposed parts. Comparison with normal palm can reveal mild cases of palmer melanosis.
- *Spotted melanosis or raindrop pigmentation:* this appears on the chest, back and extremities (57.4%) (Fig. 2).
- *Spotted keratosis:* if palmo-planter keratosis with melanosis appears in adults, it is almost sure clinical evidence of arsenical dermatosis (ASD). This starts as spotted keratosis followed by diffuse keratosis of the whole palm/soles with cracks and fissures (hyperkeratosis). Palmo-planter keratosis was observed in 61.4% of cases (Fig. 3).
- *Dorsal keratosis:* Gradually keratosis extends to the dorsum of the hands and feet and in advanced cases may extend up to the limbs and the whole body (body keratosis). Dorsal keratosis was observed in 30% of ASD (Fig. 4).
- *Leuco melanosis:* pigmented and depigmented spots side by side are sometimes seen in advanced cases (31.7%). Probably the stimulation of melanocytes in early stages results in melanosis and destruction in later phase gives rise to depigmented spots (Fig. 5).

Minor Dermatological Signs

- *Mucous membrane pigmentation:* Pigmentation of the tongue and lips may be seen (7.1%) along with melano-keratosis of palms/soles.
- *Non-pitting oedema:* this is sometimes seen (5.1%) in feet (Fig. 6).
- *Conjunctival congestion:* this is rarely observed (4%).

Non-dermatological Signs and Complications (Stage III)

Major Complications

(1) Lungs: asthmatic bronchitis (32.4%).
(2) Liver: enlarged (27.7%). Fibrosis giving rise to ascites (0.5%). Hepatic failure with jaundice rarely occurs.

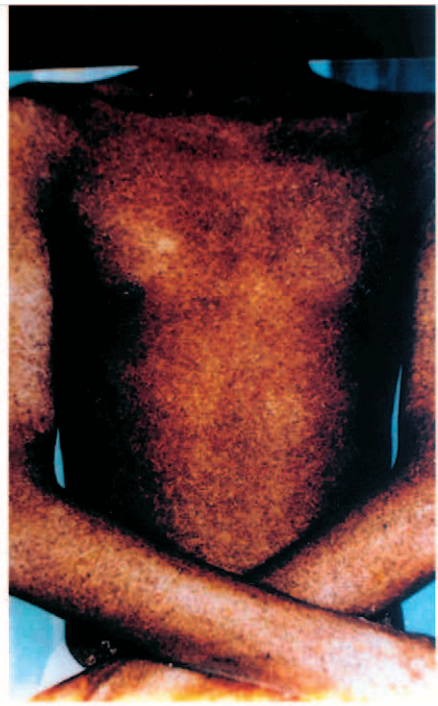
Minor Complications

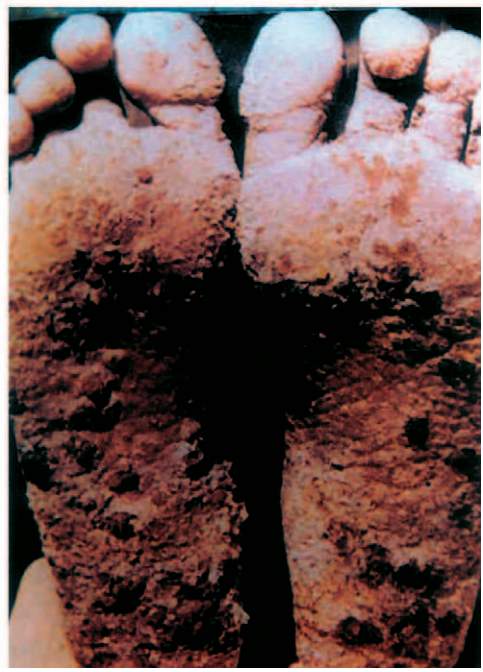
(1) Weakness and anaemia was present in 14.35%.
(2) Burning body: myalgia was present in 1.48%.
(3) Laryngitis (0.3%).
(4) Hypothyroidism (0.3%).
(5) Suprarenal deficiency (0.1%).
(6) Myopathy was seen in only one case (0.02%).
(7) Ischaemic gangrene (black feet) was observed in only one case (0.02%).

The high concentration of arsenic in mine effluents probably produced vascular damage with black feet in other countries. In West Bengal, lower concentrations of arsenic of ground water working for a long period resulted more in affection of the skin rather than the vessel wall.

Malignancy (Stage IV)

In all, 250 cases were diagnosed with cancer (5.14%) of which 212 were skin cancers (4.35%) (Figs. 7 and 8) and 38 were internal cancers (0.78%). Skin cancers were mostly squamous cell carcinoma (161 cases) and Bowen's disease (51 cases) (Fig. 9). Out of 38 internal cancers, three were bronchogenic carcinoma, three were gastric carcinoma, five were bladder affections and five were genital tract affection. It is possible that many more cases were undetected.





Upper-left: Fig. 1. Diffuse melanosis of palms (compared to normal).

Upper-right: Fig. 2. Spotted melanosis.

Right: Fig. 3. Spotted keratosis of soles.

Fig. 4. Dorsal keratosis.

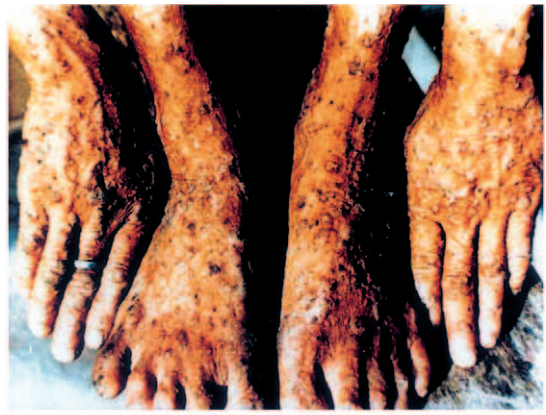


Fig. 5. Leucomelanosis.

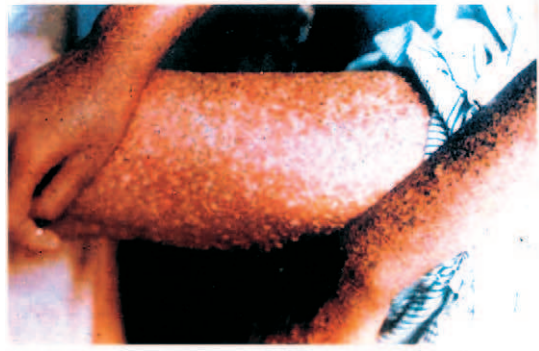
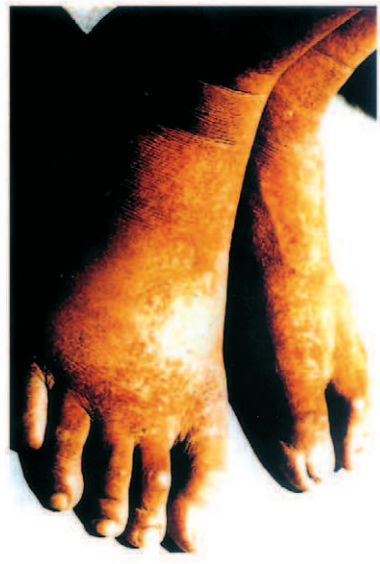
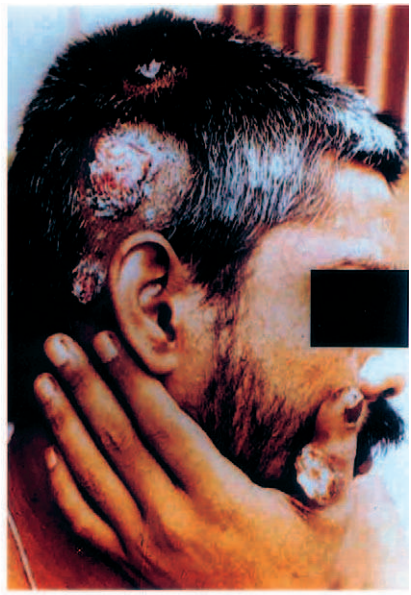
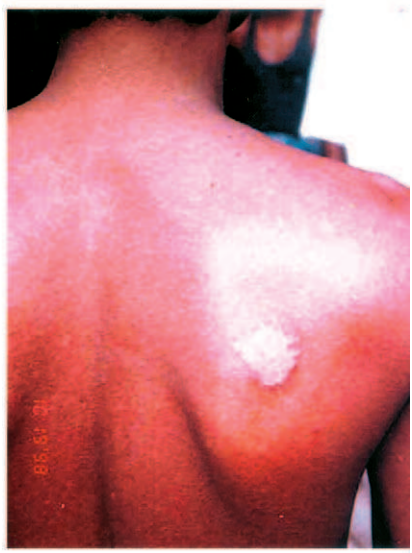


Fig. 6. Non-pitting oedema.







Upper-left: Fig. 7. Squamous cell carcinoma.

Upper-right: Fig. 8. Multi-centric skin (squamous cell) carcinoma.

Right: Fig. 9. Bowen's disease.

Increasing Arsenicosis (Table 3)

In 1983, the number of affected districts, blocks, villages and ASD patients were 4, 5, 5 and 127, respectively. During the span of 5 years from 1983 to 1987, these numbers increased to about 10 times viz. 6, 40, 61 and 1217, respectively. At that time, the district of Murshidabad was found to be maximum affected followed by the district of Nadia. During the next 12 years, i.e., from 1987 to 1999 the School of Environmental studies (SOES) and

TABLE 3

Increasing incidence of arsenicosis in West Bengal (1983–1998)

Year	Affected districts	Affected blocks	Affected villages	ASD patients
1983	4	5	5	127
1984	5	12	15	241
1985	6	17	24	485
1986	6	30	40	1068
1987	6	40	61	1217
1988	6	42	78	2026
1989	6	43	79	2185
1990	6	44	123	24,000
1993	6	47	415	83,000*
1994	6	47	428	85,600*
1995	6	54	544	108,800*
1996	7	60	966	200,000*
1997	9	63	1100	>200,000*
1998	9	69	1500	>225,000*

* Estimated figure.

TABLE 4

Districtwise arsenic-affected blocks and villages of West Bengal (cumulative 1983–1998)

Districts	No. of affected blocks	No. of affected villages	No. of ASD cases	Arsenicosis (estimated cases)
N-24 Parganas	19	445	1428	60,000
S-24 Parganas	10	147	472	30,000
Nadia	15	271	968	45,850
Murshidabad	13	428	1228	59,000
Malda	5	165	660	25,000
Burdhman	2	20	99	5,000
Hugli*	1 14	0	0	
Howrah*	3	4	0	0
Calcutta	1	6 wards	10	150
Total: 9	69	1500	4865	225,000

ASD, arsenical dermatosis.
* Not yet surveyed for arsenic patients.

the author jointly surveyed numerous villages. During the span of 12 years the number of arsenicosis patients increased more than 100 times (2000–300,000). In this period, North 24 Parganas district was found to be the maximum affected with arsenicosis (Table 4. The increase is both due to: (1) a real increase in the number of patients; and (2) increased manpower, numerous field studies and investigative facilities resulting in more patients being revealed.

Diagnosis

First step: clinical diagnosis of arsenicosis is confirmed by a high concentration of arsenic in the nails, hair, skin scales and urine along with clinical features of arsenical dermatosis (ASD), i.e., melanosis and keratosis. The subclinical stage of arsenicosis is characterised by high levels of arsenic in the nails, hair, skin scales and urine without any clinical signs of ASD. Normal arsenic levels in urine, nails and hair are: urine, 5–40 μg/day (water intake

1.5 l/day); nails, 0.43–1.08 mg/kg (toxicity level >1 mg/kg); hair, 0.08–0.25 mg/kg (toxicity level >1 mg/kg) (Chowdhury et al., 2000).

Second step: tube well water (TWW) as the vehicle of arsenic ingestion is proven by a high concentration of As in TWW, i.e., above permissible limit (0.05 mg/l, i.e, >50 μg/l) as was recommended by the WHO at that time.

Third step: underground soil. The source is proven by high concentrations of As in borehole sediment usually at 50–150 ft. depth.

Fourth step: mechanism of leaching of As from underground soil. Unlike many parts of the world, volcanoes, hot springs, effluents from copper, gold, coal mines are not the cause of contamination. The only non-geogenis source identified was a surface soak from a Paris green factory. The withdrawal of underground water in excessive quantities for irrigation and domestic purposes causes dehydration/cracks in underground strata. Probably both the pyrite oxidation theory and the iron oxyhydroxide reduction theory play a part.

CONCLUSIONS

(1) Avoid water that contains more than 0.05 mg/l (>50 μg/l) of arsenic for human consumption.
(2) Toxicity can be prevented by high protein, vitamins and selenium, but accelerated by malnutrition.
(3) Chelating drugs (BAL, D-penicillamine, DMSA or DMPS) are of little benefit in late stages, such as the complication and malignancy stages. However, melanosis could be cleared, but keratosis remains unchanged. Progress of complications may be prevented.
(4) Long duration, i.e., 6 months to 10 years (2–5 years on average) is required for development of the clinical stage of arsenicosis to show classical skin manifestations.
(5) The pre-clinical period depends on: (a) arsenic concentration of drinking water; (b) daily consumption of contaminated water; and (c) nutritional status of the person consuming such water.
(6) Source of arsenicosis: (a) exogenous (industrial) cause affects mostly the western world; and (b) endogenous (geological) arsenic affects mostly Asian countries.
(7) Misconceptions of arsenicosis as leprosy, sexually transmitted disease (STD), infectious disease etc., leading to social problems, e.g., breach of marriage engagements, divorce, service and business interruption and social isolation, could be overcome by mass education.
(8) Supply of arsenic-free water to the affected community seems to be the only long-lasting solution for the spread of arsenicosis.

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Arsenic Mobilization by DMPS

H. Vasken Aposhian, Mary M. Aposhian

ABSTRACT

The chelating agent sodium 2,3-dimercapto-1-propane sulfonate (DMPS, DIMAVAL) can mobilize and increase the urinary excretion of arsenic in humans. DMPS is a water soluble compound that can be given by mouth in the form of capsules. In the former Soviet Union and Europe, DMPS has been used to mobilize body stores of arsenic and mercury and to increase their urinary excretion. It is biotransformed to and excreted primarily as cyclic and acyclic polymers. It has both an extracellular and intracellular distribution. The pharmacology and pharmacokinetics in humans have been studied extensively. This therapeutic agent is less toxic than the lipoid soluble British Anti-Lewisite, another chelating agent. DMPS has been used as a mobilizing agent and challenge test to increase the urinary excretion of arsenic and demonstrate that there are body stores of this carcinogenic metalloid.

Keywords: DMPS; arsenic mobilization; arsenic excretion; urinary arsenic

INTRODUCTION

Arsenic compounds have a long history of use for homicides, suicides, herbicides, chemical warfare agents and treatment for pathogenic infectious agents such as syphilis. Probably no other toxic agent has been written about in literature and history as often and as long as have the compounds of this metalloid. An excellent brief recounting of these historical reports can be found in Abernathy et al. (1996). Even so, antidotes and treatment for overexposure of a human to it are available (Klimova, 1958; Aposhian, 1983; Klaassen, 1996; Aposhian et al., 1997, 2000a,b).

The modern era of treatment for arsenic toxicity began with the development of British Anti-Lewisite (BAL, dimercaprol, 2,3-dithiol-1-propanol) during World War II (Stocken and Thompson, 1949) because of the threatened use of the arsenic-containing chemical warfare agent called lewisite, which contains arsenic in the +3 oxidation state.

Trivalent arsenic can form a chelate with vicinal (on adjacent carbon atoms) thiol groups. It has a greater affinity for two such thiols than for a single thiol. A chelate is a heterocyclic compound which contains a metal or metalloid atom. This was the rationale (Peters, 1955) for the development of BAL (Fig. 1). From the 1950s to the 1980s, BAL was the drug of choice in the United States for the treatment of arsenic poisoning (Klaassen, 1996). It is still stockpiled by the armies of many nations for its anti-lewisite activity. BAL is not a perfect drug. It has many disadvantages. More than 50% of patients receiving it have unpleasant side effects (Klaassen, 1996). Since it is a liquid, it must be given by deep intramuscular injection. When it leaves the syringe, the compound itself causes pain in the tissues into which it is released. Also it has limited stability because it is readily oxidized. One of the most important disadvantages of this agent is that although it decreases the arsenic content of most tissues, the BAL-arsenic chelate redistributes the metal or metalloid to the brain (Fig. 2) (Hoover and Aposhian, 1983; Aposhian et al., 1984).

For these reasons, chemical analogs of BAL were synthesized during the 1950s by Petrunkin (1956) in the former Soviet Union. He sought a water soluble, less toxic chelating agent that could be given orally. The most effective analog (Fig. 1) was sodium 2,3-dimercapto-1-propane sulfonate (DMPS, DIMAVAL, Unithiol). It was established as an official drug of the Soviet Union in 1958 (Klimova, 1958). It did not become available to the

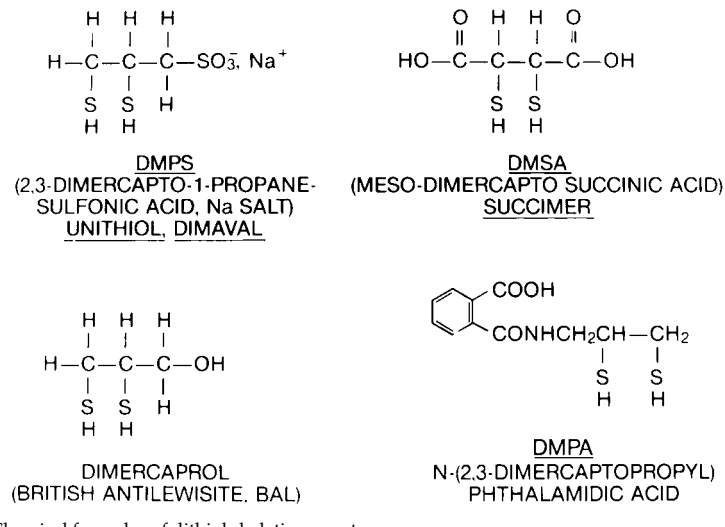


Fig. 1. Chemical formulas of dithiol chelating agents.

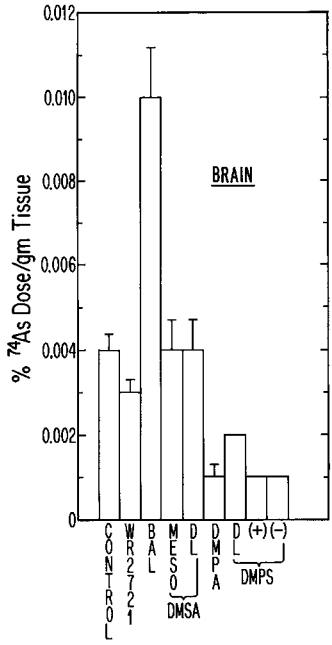


Fig. 2. Arsenic concentration of the brains of male New Zealand rabbits 24 h after administration of sodium ^{74}As-arsenite. Chelating agents was given 1 h after the arsenite.

western world until about 1978 when it was synthesized and marketed as DIMAVAL by Heyl, Berlin. It has had wide use since then in Germany and other countries.

Although a number of DMPS preparations are available, DIMAVAL is the only one prepared by acceptable western pharmaceutical procedures. A number of reviews of this and other chelating agents such as meso-2,3-dimercaptosuccinic acid (DMSA, Chemet) are available (Aposhian, 1983; Aposhian and Aposhian, 1990; Angle, 1993; Aposhian et al., 1995; Aaseth et al., 1995; Kemper et al., 1990).

THERAPEUTIC USES

DMPS has been used for treating humans with arsenic (Aposhian, 1983, 1998; Aposhian et al., 1995, 1997, 2000a, Aposhian et al., 2000b) and mercury (Clarkson et al., 1981; Campbell et al., 1986; Gonzalez-Ramirez et al., 1998), or lead (Chisholm and Thomas, 1985) toxication. It is registered in Germany for the treatment of arsenic or mercury poisoning. When compared to D-penicillamine and *N*-acetyl-DL-penicillamine, DMPS was the most effective for clearing the blood of mercury of patients with mercury poisoning in the Iraqi mercury disaster of the late 1960s (Clarkson et al., 1981). It is useful for the treatment of Wilson's disease, an inherited metabolic disease characterized by tissue accumulation of copper (Walshe, 1985).

Advantages

Both peroral and parenteral preparations of DIMAVAL are available. This has allowed data dealing with DMPS to be obtained using both the oral and i.v. preparations (Maiorino et al., 1991; Hurlbut et al., 1994). Fewer suppositions need to be made, if the pharmacokinetics of a drug are determined using data obtained after i.v. administration. The pharmacokinetic parameters are shown in Table 1 and Fig. 3. The pharmacokinetics of

TABLE 1

Pharmacokinetics of total DMPS after per oral administration of 300 mg DMPS to 10 normal young men

	C_{max} (μM)	t_{max} (h)	AUC (μM × h)	$t_{1/2}$ (h)
Blood				
Mean	11.9	3.7	148	9.1
± SE	1.5	0.7	11	1.3
Plasma				
Mean	25.3	3.4	318	9.9
± SE	3.0	0.6	21	1.6

Other conditions for this table can be found in Maiorino et al. (1991).

DMSA, on the other hand, are limited since the data were obtained using capsules for oral administration, the only preparation available (Dart et al., 1994).

This drug has been widely used in humans in the Soviet Union (Klimova, 1958), Germany and in the United States (Aposhian et al., 1995). In the United States, in fact, there has been a relatively large DMPS 'gray' market. People who think they have mercury toxicity from such sources as dental amalgams obtain the drug from compounding pharmacists to mobilize and increase the urinary excretion of mercury. The number of such people is quite large. Because a new drug application has not been submitted to the FDA for DMPS, this drug does not have FDA approval for its use in the USA. It is unfortunate that toxicological data including possible efficacy and side effects have not been compiled and published because of the questionable legality of DMPS sales and use in the United States.

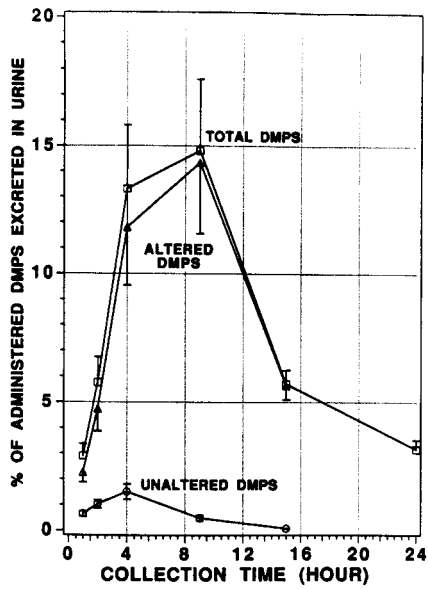
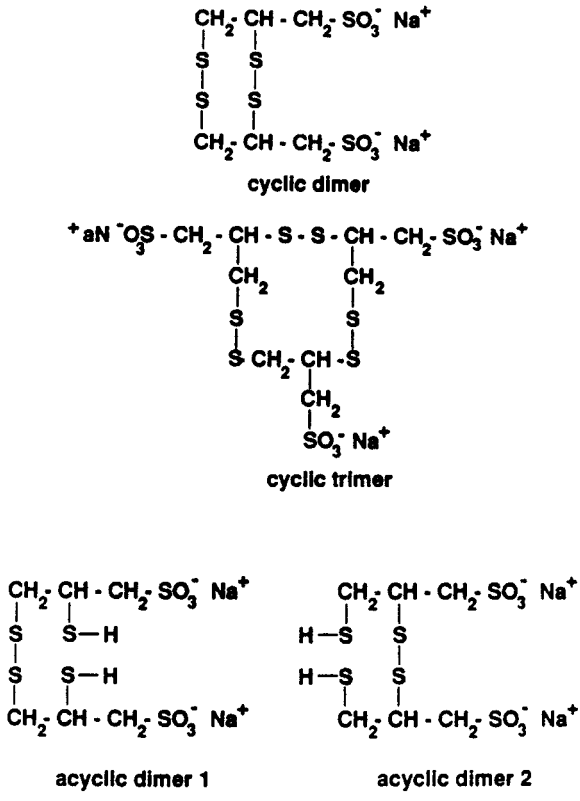


Fig. 3. Urinary excretion of total DMPS, altered DMPS and unaltered DMPS by 10 fasted males given DMPS (300 mg) p.o. Urine samples were treated immediately with bromobimane for unaltered DMPS. Total DMPS was determined by treatment with DTT followed by bromobimane derivatization. Altered DMPS is total DMPS minus unaltered DMPS. Each point is the mean ± SE.

Fig. 4. Biotransformants of DMPS found in urine.



Metabolism

DMPS is biotransformed by humans to acyclic and cyclic disulfides polymers (Maiorino et al., 1996) (Fig. 4). These are the predominant forms found in the urine. Our laboratory has shown that whereas DMSA is biotransformed almost completely into a mixed disulfide with cysteine (Maiorino et al., 1993), a DMPS–cysteine adduct is not the major excretory form found in the urine (Maiorino et al., 1996).

Distribution

Another difference between DMSA and DMPS is that the latter has intracellular distribution as well as extracellular. Biliary excretion of DMPS was found (Zheng et al., 1990) when DMPS was given to rats intravenously (Fig. 5). This is the major evidence for its intracellular distribution although it was suggested by earlier work with red cells (Reuther et al., 1982). When DMSA was administered, it was not detected in the bile confirming that it does not have an intracellular distribution (Zheng et al., 1990).

Precautions and toxicity

Hypotensive effects are possible when DMPS is given parenterally (Klimova, 1958). The intravenous use of this chelating agent should be reserved for the treatment of acute poisonings since oral DMPS preparations are available for all other uses. If the patient has a history of allergies to penicillin, sulfonamides and/or other sulfur-containing drugs, neither DMPS nor DMSA should be used.

The LD_{50}s of these chelating agents (the amount that will kill 50% of the animals) have

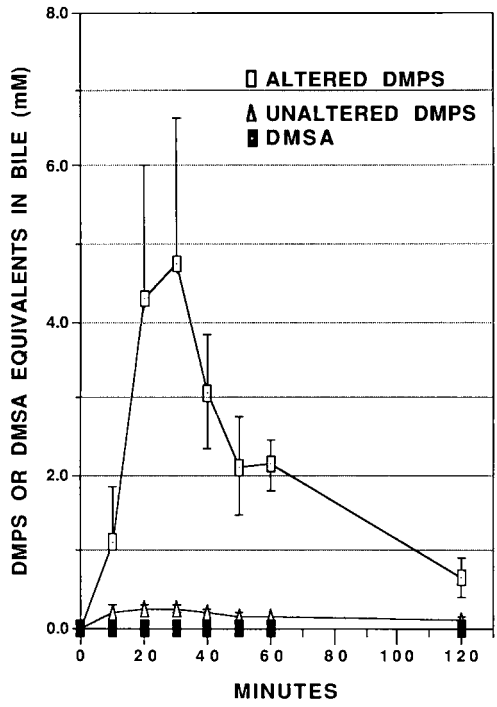


Fig. 5. Rats were injected i.v. with DMPS or DMSA and the bile collected and analyzed for DMPS and DMSA.

TABLE 2

LD_{50} determination, i.p., in mice

Compound	LD_{50} (mmol/kg)	95% confidence interval
BAL	1.48	1.11–1.97
DMPS	6.53	5.49–7.71
meso-DMSA	13.73	11.36–15.22

been determined (Table 2) in mice (Aposhian et al., 1984). (The larger the LD_{50} value, the less the toxicity). Although the LD_{50} of DMSA is about twice that of DMPS in mice, this has very little significance since the therapeutic doses are much smaller on a body weight basis.

USE OF DMPS TO INCREASE ARSENIC EXCRETION

Our laboratory has developed a DMPS mobilization protocol for arsenic (Aposhian et al., 1997, 2000a,b). The results give a better approximation of the body burden of arsenic. It is an approximation, not an absolute value. Perhaps a better term would be hidden arsenic rather than body burden. The procedure for the mobilization protocol is as follows:

(1) Fast overnight for 11 h and collect urine during this time. The purpose of the fast is to decrease the possibility that metals in the GI tract will combine with the administered DMPS and thus decrease its effectiveness.

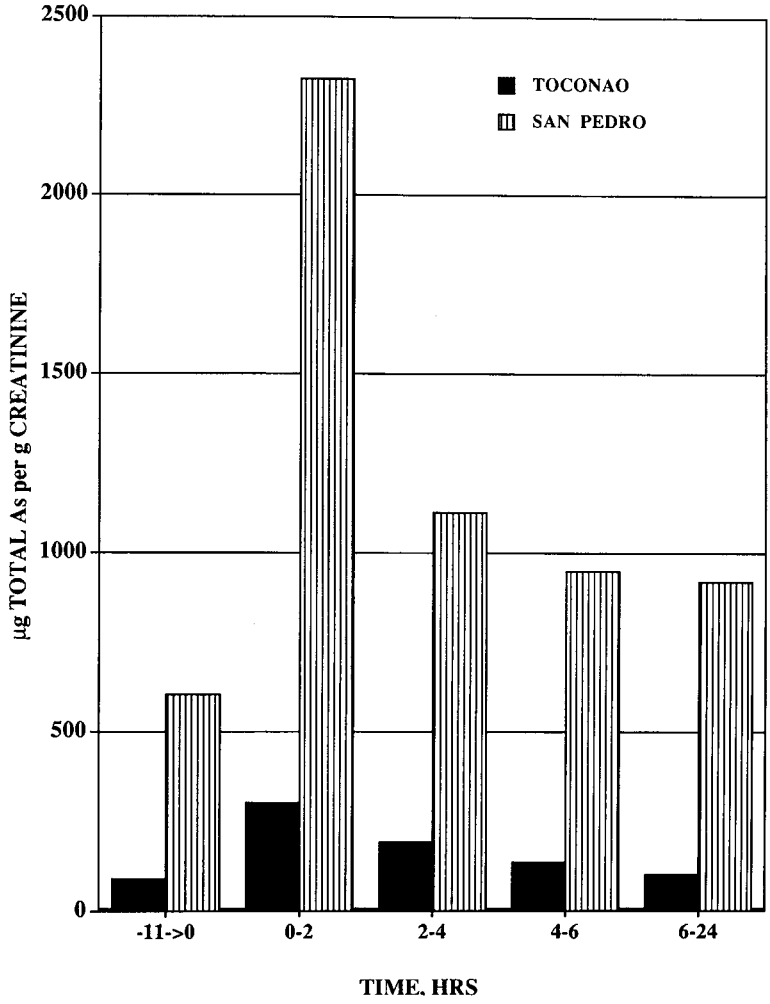


Fig. 6. Total arsenic concentration in urine before and after administration of 300 mg DMPS. DMPS was given per oral at time 0. Error bars represent ± standard error.

(2) Next morning at 0 h give 300 mg DMPS p.o. and collect urine for desired time periods.
(3) At +4 h, a sandwich, chicken or turkey, is eaten to relieve the fast.
(4) At a predetermined time, end urine collection. Usually urine is collected for 8 h for the DMPS arsenic challenge test.

The urines have been analyzed for arsenic by hydride generation-atomic absorption spectrometry (Aposhian et al., 1997) or HPLC– hydride generation–atomic fluorescence (Aposhian et al., 2000a,b) using the method of Le et al. (2000). After DMPS administration, there were increases in all the arsenic species in the urine (Figs. 6 and 7) (Aposhian et al., 1997). The mobilization test was originally devised to determine hidden mercury (Cherian et al., 1989) but now has been modified as a mobilization protocol for arsenic (Aposhian et al., 1997, 2000a,b). In addition, the use of DMPS in arsenic-exposed humans resulted in the first evidence that monomethylarsonous acid (MMA^{III}) was excreted in the urine of

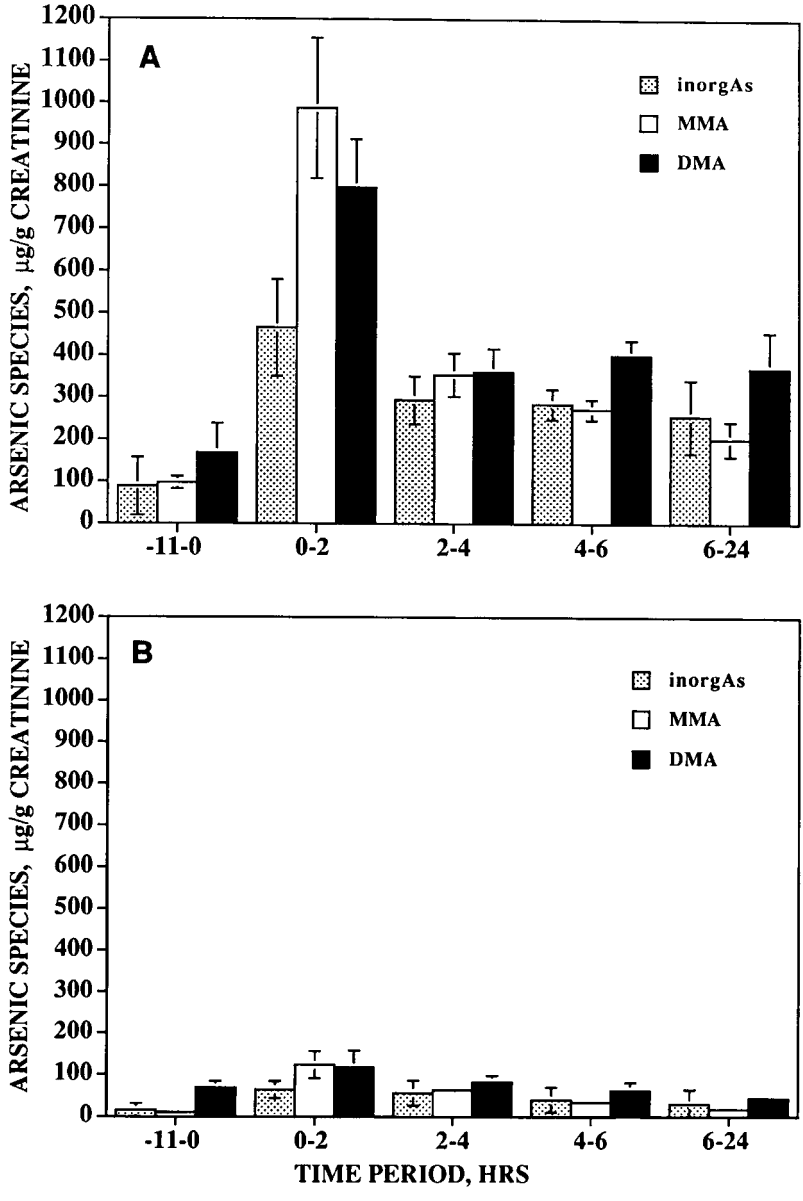


Fig. 7. Concentration of arsenic species in the urine, μg/g creatinine, before and after 300 mg DMPS administration per oral. (A) San Pedro de Atacama subjects. (B) Tocano subjects.

humans (Aposhian et al., 2000a). The reader is referred to the chapter in this monograph by Dr. Guha Mazumder on the use of DMPS in treating people in India chronically exposed to arsenic from drinking water containing very high concentrations of arsenic.

One should not forget, however, that the best and most reasonable procedure for handling humans exposed to arsenic is to remove them from the site of the exposure or

prevent the exposure from occurring in the first place. Unfortunately, this is not always possible.

In summary, DMPS has had extensive use in humans as a chelating agent. In this manner, it can increase the urinary excretion of arsenic species and thus decrease the body burden of this dangerous, highly toxic and carcinogenic metalloid.

ACKNOWLEDGEMENTS

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Arsenic Mitigation and Water Supply in Bangladesh

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ABSTRACT

The arsenic problem has become a big challenge for several countries. One of the main and major problems is to address water-supply-related issues in a way which is appropriate to Bangladesh and to other similar developing countries. The groundwater of Bangladesh is heavily contaminated with arsenic. The arsenic problem in Bangladesh is by far the worst in the world. This paper reviews the situation existing in the country. About 30 million people are exposed to ingestion of arsenic-contaminated tubewell water exceeding the Bangladesh standard of 50 ppb. More than 80% of the districts have one or more arsenic-contaminated tubewells. About 95% of the Bangladesh population drink tubewell water. Almost no progress has been made with the supply of safe water to the affected people. Inadequate efforts have been undertaken to communicate the mitigation knowledge or to address the problem in a collective way. Lack of coordinated and collaborative activities, recognition and use of available information, planned and prioritized initiatives, appropriate mass communication, integration of the stakeholders, consideration of the alternative water sources, and availability of appropriate techniques and technologies have slowed down arsenic mitigation initiatives.

Keywords: stakeholders; awareness; coordination; testing; option; community

INTRODUCTION

Access to drinking water in sufficient quantity and of a quality equal to basic needs is a fundamental right of all people. The International Conference on Primary Health Care, the International Drinking-Water Supply and Sanitation Decade, the International Ministerial Conference on Children Rights, the Rio Summit in 1995 and almost all other public health-related meetings reiterated the inevitable roles and needs of drinking safe water in human life. In spite of this, unfortunately more than a billion people lack safe water at the end of the 20th century.

About 95% of the approximately 120 million people drink tubewell water in Bangladesh drawn from alluvial aquifers underlying the Ganges and Brahmaputra delta (UNICEF, 1999). In Bangladesh, surface water is widely available, but it is heavily polluted with fecal matter and other pollutants, and also in some areas runs into short supply during the dry season. Intensive efforts were invested over the last three decades in bringing about the changes required to make groundwater available and its use through tubewells. However, the effectiveness of this remarkable achievement has been challenged by the recent discovery of massive arsenic contamination of groundwater. Arsenic contamination of groundwater, and to a lesser extent, surface water has been observed in many countries of the world (DPHE, 1999). Drinking/ingestion of arsenic-contaminated water/air is associated with hyperpigmentation, keratosis, skin cancer, neurological problems, reproductive health problems, cardiovascular diseases and cancer in various organs (Karim, 2000; Smith et al., 2000). The arsenic problem in Bangladesh is by far the worst in the world (DPHE, 1999). For Bangladesh, the challenge is to deal with it in an appropriate way, giving due consideration to both short-term and long-term issues. The country is overburdened with: one of the highest population densities of the world (826 persons/km^2); low GNP (estimated per capita US $230); high infant mortality (66 per 1000 live births); high under-5 mortality rates (112 per 1000 live birth); high illiteracy (53% for people 7 years of age and above, 41% for men 15 years and above and 58% for women 15 years and above); and other related problems (UNICEF, 1999).

There are several millions of tubewells in Bangladesh. The cause of the contamination is geological in nature, although opinions vary on the precise mobilization mechanism (Nickson et al., 1998; Nickson et al., 2000). As many as 30 million people may be actually exposed to ingestion of arsenic-contaminated tubewell water exceeding the Bangladesh standard of 50 ppb (the WHO provisional guideline is 10 ppb) while, due to the limited extent of testing, more than 60 million are at risk (Zones, 2000). The people of this small developing country are fortunate that extensive global and national attention/inputs are being given to mitigate the problem. However, the reality is that there has been negligible improvement in the supply of safe drinking water to the affected people even after about several years of wide information about the problem.

Here we present our assessment of the ongoing mitigation and outstanding issues in Bangladesh. We believe that sharing of this information will contribute toward more appropriate planning and implementation of mitigation actions in Bangladesh and in similar situations elsewhere.

EXTENT AND MEASUREMENT OF ARSENIC CONTAMINATION IN BANGLADESH

The most important countrywide water analyses include surveys undertaken by the Department of Public Health Engineering (DPHE) with assistance from UNICEF, and DFID, plus independent surveys by DCH/SOES and NRECA (NRECA, 1997; DPHE, 1999; DPHE/UNICEF, 1999; Zaman et al., 1999; Government of Bangladesh et al., 2000). During these initiatives agency-based sampling, patient-based sampling, and exposure-based assessment of the water samples were carried out using both field kits and laboratory

TABLE 1

Comparison of selected information on the nature of the problem

		(A) DPHE–UNICEF	(B) DPHE (BGS/MML)
1	Survey period	1997–1998	1998–1999
2	Technique used for water analyses	Kit	Laboratory
3	Number of tested wells	22,777	3,500
4	Number of district surveyed	64	61
5	Number of district with up to 10% contaminated tubewells	30	22
6	Number of districts with >10% <40% contaminated wells	25	31
7	Numbers of districts with >40% contaminated tubewells	9	8

methods of analysis. About 85% of the country has been surveyed; almost all districts indicated the presence of arsenic content over 50 ppb in one or more tubewells. The extent of contamination in deep wells (more than 200 m deep) was observed to be about 3% by both the BGS/MML and NRECA surveys. This statement must be qualified by the fact that most deep wells sampled were from the coastal regions where the wells have been sunk to avoid salinity in the shallow aquifer.

All surveys indicated that the southwest region is extensively contaminated. Shallow tubewells are the main source of the arsenic problem. We are not aware of examples where the survey results varied substantially in places with about a quarter of the tubewells contaminated at the 50 ppb level. Table 1 shows that two different surveys, using different methods at different periods, provided similar results.

Overall, information about the worst affected areas where millions of people may not have access to an arsenic-safe tubewell within a convenient distance is available from 1997. Efforts to supply safe water and/or to launch mass communication about the short- or long-term measures to the people in the worst affected areas are yet to be seen.

METHODS USED FOR TESTING TUBEWELLS

Tubewells are being tested/screened by various agencies, using different field-kit and laboratory methods. Most of the used field kits showed problems in measuring the arsenic content below 50 ppb. It is obvious that both field kit and laboratory methods should be used to measure arsenic; repeating laboratory tests for a small appropriate portion of the field kit-tested samples. Although the measurement of arsenic is one of the most important strategies, significant development has not been observed in the development of field kits or in access to reliable laboratory facilities. Some limitations in the technical and operational qualities of the methods used have been recognized repeatedly since 1997 (NRECA, 1997; Hoque et al., 1998; NEERI, 1998; DPHE, 1999). A number of recommendations have been made many times, and are worth repeating:

(1) field kits should be standardized;
(2) field kits should be produced locally to suit local conditions;
(3) appropriate laboratory facilities should be made accessible for proper quality control;
(4) testing by field-kit or laboratory method should be controlled qualitatively through surveillance by a competent authority;
(5) wide dissemination, coordination and collaboration about the data should be practised by all concerned stakeholders.

SAFE WATER OPTIONS

Mitigation options involve both arsenic-avoidance and arsenic removal. Lack of knowledge about safe water options has hindered the mitigation initiatives. Arsenic-safe sources

include surface water and rainwater as well as arsenic-safe groundwater sources. It has been reported that the proportions of As(III) and As(V) as well as iron and other elements, such as manganese and boron, in water vary with location (NRECA, 1997; DPHE, 1999; Government of Bangladesh et al., 2000). All these factors influence the type and performance of arsenic-treatment options (NRECA, 1997; Government of Bangladesh et al., 2000). The appropriateness of household or community-based arsenic treatment options or any water supply option will vary according to the local social and physical conditions. So the feasibility study/research of appropriate water supply will be required to consider various combinations. Such appropriate national initiative has been undertaken from September 2000 only (after the conference), and that too is looking onto the arsenic removal technologies.

However, small-scale research has presented indicative data about potential water supply options from 1998 (NRECA, 1997; Hoque et al., 1998; DANIDA, 1999; DPHE, 1999; Hoque et al., 2000). Water supply through deep hand-tubewells (>150–200 m), sharing of arsenic-free shallow tubewells, pond-sand-filtration of surface water, rainwater harvesting, boiling of surface water, alum treatment of arsenic, packeted chemical treatment ('tea bags'), and removal of arsenic by storage at household level (passive sedimentation) have been studied by more than one organization and promoted by government and some partner agencies as appropriate options. Although research initiatives have indicated doubts about performance/appropriateness of some of these options, such as removal of arsenic by 24-h storage (passive sedimentation), 'tea-bag' treatment and alum treatment at household level and disseminated the findings at national policy and program levels, those options are being promoted. Hand-deep tubewells are costly and often not affordable by the individual families. This option is in high demand by the people.

The facts and indicative results about the potentials of the alternative sources such as rain water, dugwell water and surface water (with some treatment) have been neglected. Expensive but reliable water supply mechanisms through community participation, cost sharing, and private organizations should be explored. Lack of interest in collecting and reviewing available information about water supply options, and then building on the collective knowledge for proper research or other development have worsened the situation.

INITIATIVES AT LOCAL LEVEL

In general, awareness about arsenic contamination of groundwater and its consequences for human health is low (Hoque et al., 1998; Mitra and Associates, 1998; Asiatic Social, 1999; DPHE, 1999). This applies to government, NGOs, and the local people. A survey conducted in 1998 indicates that only 7% of the 1839 women interviewed had heard of arsenic (Mitra and Associates, 1998). Results of another evaluation showed that in arsenic-mitigation project areas, information on the test results and precautions was not necessarily disseminated to the user population and no standardized procedure was followed to mark the tested tubewells (Asiatic Social, 1999). In addition, there was a general resistance to change water consumption and water management behaviour. It was also reported that there were areas where tubewell after tubewell have been marked 'red' and no surface water or other arsenic-safe source of water is available. Another report indicated that water options with little/no merit to remove arsenic were promoted at community level even after repeated dissemination about the unacceptable performance of those options (NRECA, 1997; Hoque et al., 1998). It was also observed that the people were annoyed and confused when corrected information was communicated to them after receiving incorrect information (Hoque et al., 1998). However, people's willingness (community and elected local political leader involvement) to plan and implement arsenic mitigation has been observed in an applied research model (Hoque et al., 1998).

Many initiatives such as those by DPHE–UNICEF, DPHE–UNICEF–NGO, UNDP funded Ministry of Health and Family Welfare Project, World Bank funded Bangladesh Arsenic Mitigation Project by the Government, DPHE-Danida, Water-Aid, NGO-Forum, Rotary, Dhaka Community Hospital, and other UN, donor and NGO implemented mitigation projects are in progress and the level of awareness may have improved in those few sub-districts only. We could not locate that information. However, those initiatives still do not cover most of the affected or even 'hot spot' areas.

DISCUSSION

It is encouraging that almost all stakeholders (except the rural population) are sensitized to address the problem. The reality is that almost all the millions of affected people are yet to be supplied with arsenic-safe water. Most of the tubewells have yet to be tested. Some people in some affected areas might have started sharing arsenic-safe tubewells from contaminated ones, provided there were arsenic safe tubewells in the neighbourhood and people were informed/aware about it. But that information too is not properly documented. Although lack of available and/or appropriate field kits and lack of knowledge about appropriate water supply options exits, the people could be helped significantly through coordinated and collaborative efforts. Nonetheless, we cannot understand why there has been a lack of proper use of available knowledge in building collective efforts to address the problem. An acceptable and appropriate agenda should have been (and is still needed) communicated at all levels to address the situation in emergency, intermediate and long-term perspectives.

Initiatives for integrating arsenic mitigation with health, education, agriculture, environment and other sectors should be immediately given due consideration. The aspect of gender-responsive mitigation, particularly the involvement of women in mitigation, has been neglected. From the point of view of sustainability, cost-effectiveness and speed of installation, community level supplies have obvious advantages in badly contaminated areas.

CONCLUSION

Overall, tens of millions of people are drinking arsenic-contaminated water and effective mitigation in Bangladesh is yet to be seen. Lack of coordinated and collaborative activities, recognition and collective efforts, use of available information, proper information-sharing, planned and prioritized initiatives in mass communication, integration of the stakeholders, consideration of the alternative water sources, building of local capacity and availability of appropriate techniques and technologies have hampered arsenic mitigation initiatives. Appropriate research has not been adequately encouraged to contribute to bridging the gaps in knowledge between mitigation needs and their present scope.

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Community-Based Management of Arsenic-Affected Patients

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ABSTRACT

In a recent survey, 7000 rural people in Bangladesh were identified as having arsenic poisoning caused by drinking arsenic-contaminated tubewell water. There may be as many as 85 million people at risk. The early symptoms of arsenic poisoning, lesions on the palms and soles, disable people from working. Body lesions may also appear in the early stages causing social rejection. To get medical help many villagers sell all their assets, financially ruining themselves. These patients require long-term medical support, an arsenic-free, safe drinking water supply and economic and social rehabilitation. A unified, community-based approach of primary, secondary and tertiary health care, with social and economic components that will adjust to individual andcommunity needs, is required to achieve patient care. The Dhaka Community Hospital (DCH) intervenes at the village level by introducing a community-managed mitigation programme, run by a village committee and staffed by community workers employed from within the village. DCH acts as a guide and resource organisation. The community is responsible for determining, maintaining and monitoring a safe water supply. Improvements in community health and nutrition are promoted. Patients are provided with home-based palliative treatment. Specific treatment is to minister local lesions with ointments, antibiotics and anti-allergic medications. Special treatment is need-based. Social and economic rehabilitation is undertakenwith community participation. Community financing makes the project accountable to local people. Experience has shown that community management and ownership produce practical, affordable and sustainable programmes, vitally necessary with such a long-term problem.

Keywords: arsenic; community; community approach; health care; patient management

INTRODUCTION

Most Bangladeshis live in rural areas where shallow tubewells are their main source of water. Many of these tubewells are now known to be contaminated with arsenic. The exact number of contaminated tubewells is unknown but it is estimated that more than 85 million people are at risk. In random surveys conducted up to June 2000, arsenic lesions had been found on 7000 villagers (confirmed by biological testing of hair, nails, urine and skin by atomic absorption spectrophotometer testing). Although there is no certain staging of arsenicosis, melanosis lesions are initially seen on the trunk and advanced stages of keratosis usually appear on (but need not be confined to) the palms of hands and soles of the feet. Some keratosis lesions are pre-cancerous but the time period for carcinogenic changes to occur is not established. Patients with carcinogenic gangrene were found, including children less than ten years old. The surveys did not look for the internal cancers associated with arsenicosis. Apart from the general malaise caused by chronic poisoning, lesions disable people from working and cause social problems. When a family member is affected villagers begin to sell any assets (livestock, home, land) they have to pay for treatment, until they become financially ruined. Treatment is symptomatic; there is no specific remedial treatment other than to drink arsenic-free water.

Health care in Bangladesh is institution-based. The country is administratively divided into districts, then thanas and finally unions. The capital, Dhaka, has a full range of specialist and teaching hospitals; major cities have teaching hospitals; towns have general hospitals and so on down to union health sub-centres, each covering a population of about 20,000. They are poorly maintained and staffed. The rural road and communication networks are inadequate and so most villages are remote from any organised health care. The result is that untrained, self-styled private village 'doctors' provide 85% of Bangladesh health care. Water, sanitation, maternal and child health care and immunisation services are provided by donor-funded non-government organisations (NGOs). A recent policy change is being introduced based on building community clinics for each 6000 population. The services are not demand led, do not aim to target community health needs and provide no community access to decision making processes.

Considering the geographical extent of the arsenic poisoning, the isolated locations and poverty of the villages, the long-term nature of arsenic contamination and arsenicosis and its social and economic implications for the individual, household and community, a comprehensive need-based approach centred on the community is imperative. Primary, secondary, tertiary healthcare must be integrated with social and economical components and the whole approach must be flexible to adjust with community needs as they change. The village community itself must be able to determine its own course of action because ultimately each community has to take long-term responsibility for combating the effects of the contamination. Importantly, the care system must be affordable to be sustainable.

This article describes an approach to how an economically poor country with the associated infrastructure problems of deficient health, education and communication systems can come some way to coping with a potentially enormous public health disaster by using and building on available resources.

METHODOLOGY

Community-Based Management

Bangladeshi villages are usually quite small (less than 200 households). Although there is some economic migration to cities, this only involves one or two family members and is often seasonal. There is little migration to villages. Therefore most village households have known and lived with each other for generations, knowing each other's qualities of neighbourliness and economic and health status. Thus the concept of a 'community' is a

reality that can be used to implement health, or any other relevant, programmes. When a village approaches the Dhaka Community Hospital (DCH) for a health care programme, the DCH does not try to impose an institution-based health system but instead helps create a system whereby the community provides its own long-term integrated health care to deal with all the effects of arsenicosis. This involves not only medical but also social and economic rehabilitation. Therefore the initial discussions, firstly between the DCH and community representatives (the respected, elected and selected) and then public meetings, are a discovery phase to understand how the community sees its problem and what it currently does to solve the problem. If it is agreed that a programme should go ahead, a community committee is formed and the DCH explains its role to provide facilitation, guidance and experience and the community's role of organising, managing, taking responsibility for and thus owning the programme.

Community Workers

One of the first tasks for the community is to appoint community workers who receive DCH training in arsenic health work. They are the core of the programme for it is their local knowledge and access to households that makes the health care system work. They have little problem in conducting house-to-house surveys to identify patients and motivate them to visit the doctor. They impart credibility to the mobilisation and motivational work necessary in primary health care to change water use behaviour. Once established the community committee determines how to raise money via insurance schemes to pay their salaries.

Community Physician

The community physician is initially employed by the DCH, who trains him. Bangladeshi medical schools prepare doctors for working in institutions in the towns and cities, so initial DCH training is to orient the doctor for village level work within a community setting. He is taught to identify and manage arsenic patients and the importance of safe water management techniques. He is also taught how to monitor and conduct medical audits and about the supervisory role he has in the system. He receives medical backing by specialists from the DCH who regularly visit the programme areas to see referred patients and assist with any other problems. Gradually as the programme progresses, the community provides his funding, so that the doctor becomes an employee of the community. In some areas, one community physician may serve several villages and his salary is raised jointly among the villages.

Water Management

As the primary source of arsenic poisoning is the use of tubewell water, the initial primary health requirement is to stop exposure. Tubewell testing and regular re-testing is part of the community workers' work. Current user-safe field test kits are insufficiently sensitive to guarantee safe water and laboratory testing facilities are scarce and too expensive to be undertaken regularly. Also, it is unknown whether further deterioration and contamination in groundwater quality will occur through continued extraction particularly with the continued use of shallow pumps for intensive irrigation and so the DCH promotes the use of community-managed surface water through indigenous methods. These include covered dug wells, pond sand filters and rain water harvesting. The community committees organise and form water committees that are responsible for selecting community water sites, maintaining the water source and monitoring the safety of the water. The DCH provides guidelines for these activities especially to promote equitable access to the source.

Motivation and Mobilisation

The DCH facilitates awareness campaigns that are carried out by community workers, schoolteachers, community leaders and peoples' representatives. The campaigns are used

to promote community ownership and management. A major element is to motivate the population to switch to safe water and sanitation practices and to reassure that arsenicosis is a non-contagious but debilitating disease. Campaigns are carried out through public meetings, school visits, drama, folksongs and other village-based activities.

Patient Management

(1) Patients are identified and registered in two groups: (a) people who are showing arsenic lesions, and (b) people who are not showing any lesion but drinking arsenic-contaminated water. Both groups are examined and a personal medical profile recorded.

(2) Samples of blood, nail, skin and urine are taken and sent for laboratory testing to confirm diagnosis.

(3) Home-based palliative treatment is given to improve the general health and nutritional status of the patient. This includes vitamin supplements and dietary counselling based on what is available locally.

(4) Specific treatment is used to treat local lesions: for keratosis lesions salicylic acid ointment is prescribed. Anti-oxidant tablets, antibiotics and other appropriate medication are also used under the doctor's supervision, when necessary.

(5) Special treatment is need-based. Patients requiring tertiary care, operations, excisions, amputations, limb fitting, physiotherapy and specialist care, are referred to the DCH or local hospitals if they are able to supply the required treatment.

(6) The community develops economic and social rehabilitation strategies. In a small community, the community has the liability of economically supporting destitute households and so it is to the community's advantage if patients who are unable to work or continue their occupation, are re-trained for new occupations, such as guarding new water sources, counselling new patients and community work. These jobs are paid by community contribution. Not all patients are physically fit enough to work but an improvement in their social condition is made through the awareness and motivation campaigns. The community also participates in supporting and regularly monitoring patients and at risk family members to see progress and changes.

RESULTS AND DISCUSSION

We have been facilitating community-based integrated health care programmes for over twelve years and it has been relatively simple to add a safe water component to these existing programmes. However, we are now working with nearly 300 villages in new areas, only with the arsenic care component and although this represents only a small number of Bangladesh's villages, the results achieved are encouraging. We find that the advantages of the community approach are that more patients are being identified, followed-up and supported in their communities instead of being stigmatised and isolated. We have not yet recorded significant changes in lesions through reduction in exposure but the patients who are using arsenic-safe water subjectively report feeling better. The rehabilitation component succeeds once the community understands that the lesions are not caused by TB or leprosy and that the disease is not contagious.

In villages where there are contaminated tubewells but no patients (according to the definition that patients show arsenic lesions as well as body burden) we have concentrated on promoting a change to safe surface water, to prevent exposure. We do not know why lesions are not seen in these villages; whether the tubewells have become contaminated recently, how much contaminated water individuals have drunk, or whether there are other factors involved. Yet even in these villages, after one or two community dug wells were opened through the programme, more were opened through private initiative. There have been no reported increases in diarrhoea diseases in any of the villages.

We have not yet found a village unwilling or unable to select suitable community

workers and whereas the doctors are almost always male, the community health workers are usually female. We have found that they do not have to be highly educated to perform their work to a high standard and they are particularly appreciated for enabling patients to be cared for in their own homes whenever practicable.

Motivation and awareness campaigns have been crucial for the success of the programme. It takes more time to motivate a change in water use where there are no manifestations of arsenicosis and it is the community commitment that sustains the programmes. It is particularly difficult when a tubewell used for commercial reasons is contaminated and only community action prevents its use.

Community financing makes the project accountable to the people and promotes community ownership. This encourages sustainability. UNICEF sponsored the DCH for six months to implement the programme in 170 villages. Just over eighteen months later the communities are continuing the programmes without external financial support.

One foreseeable problem is that if the number of patients requiring tertiary care increases sharply, it will be very difficult for any community to raise hospital fees. At present the DCH is able to treat patients through its extended payment schemes and community support.

CONCLUSION

By providing basic training to locally selected people, it is possible to manage arsenicosis patients in the community and with community support this reduces the individual economic burden of illness. It is also possible through creating awareness about arsenic to prevent exposure by motivating a change in water use from groundwater to safe surface water, using methods that are acceptable, affordable and sustainable. The government is unable to provide sufficient health services for its population and it will be very difficult for it to respond quickly to the arsenic crisis with institution-based solutions in all the affected villages of Bangladesh. Individual communities, with a little facilitation are able to determine, sustain and maintain their own health care strategies and this empowerment does provide improved care for arsenicosis patients and those suffering from chronic arsenic poisoning.

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Arsenic Exposure and Health Effects IV
W.R. Chappell, C.O. Abernathy and R.L. Calderon (Editors)
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A Comprehensive Review of Low-Cost, Tubewell Water Treatment Technologies for Arsenic Removal

Susan Murcott

ABSTRACT

Arsenic contamination of groundwater is the cause of a massive public health crisis in some developing countries, including Bangladesh and West Bengal, India. Remediation technologies include tubewell water treatment, surface water treatment, rain water collection, construction of deeper tubewells and groundwater treatment/aquifer recharge. The objective of this paper is to provide a comprehensive review of low-cost, tubewell treatment options, based on actual laboratory, pilot and full-scale experiments and applications. In February 1999, the author presented a preliminary review of appropriate remediation technologies for treating tubewell water in Bangladesh at the 'International Conference on Arsenic in Bangladesh Groundwater', Wagner College, New York. Twenty-two specific technologies were presented under the general categories of oxidation, coagulation/precipitation, sedimentation, filtration, adsorption, solar distillation and membrane processes. This new paper will update the earlier information for the original twenty-two technologies and provide new data on solar oxidation, microbiological approaches and new variations on processes previously covered. A summary table will present each of these technologies in terms of three criteria: performance, cost, 'appropriateness' or 'social acceptability'. The criterion 'social acceptability' goes beyond the characteristics of the technology to engage the users of the technology. An 'appropriate technology' is one that is accessible to community and women groups, those who will be the front-line users of arsenic removal technologies in Bangladesh and elsewhere. The last section of this paper discusses women's role in appropriate technology projects based on the author's experience in Nepal, India and Haiti.

Keywords: arsenic remediation technologies; appropriate technologies; tubewells; Bangladesh; developing countries; Web database

INTRODUCTION

Arsenic contamination of groundwater is the cause of a massive public health crisis in some developing countries, including Bangladesh and West Bengal, India. Remediation technologies include tubewell water treatment, surface water treatment, rain water collection, construction of deeper tubewells and groundwater treatment/aquifer recharge. The objective of this paper is to provide a comprehensive review of low-cost, tubewell treatment options.

Why focus on tubewell treatment? More than 95% of Bangladeshis (114 million people) obtain their water supply from tubewells (WHO, 1999). How many of these tubewells show contamination with arsenic? The arsenic testing program of the UNICEF-funded Bangladesh Department of Public Health Engineering (DPHE) is the largest testing effort to date in Bangladesh. DPHE has analyzed tubewell water in 61 out of 64 districts using field test kits and found detectable levels of arsenic in 29% of wells (UNICEF, 1999). This suggests that 33 million people are potentially exposed to toxic effects from drinking tubewell water.

How does an affected person, an engineer, an aid organization, an entrepreneur, or concerned citizen know the options to remove arsenic from tubewell water? More importantly, how can one compare the performance, reliability and cost of such systems? It is not a trivial task to compile these data. Until now there has not been a Web site exclusively devoted to arsenic remediation technologies for Bangladesh and other developing countries and no comprehensive database, although there are a number of excellent Web sites generally covering the arsenic crisis in Bangladesh, for example: the Arsenic Crisis Information Center: http://bicn.com/acic; the Harvard Web site http://phys4.harvard.edu/~wilson/arsenic_project_main.html; the Bangladesh Arsenic Mitigation Water Supply Project: http://www.bamwsp.org. The goal of this paper is to provide comprehensive and accurate information for tubewell treatment for Bangladesh and other developing countries, and to establish a Web site exclusively devoted to arsenic remediation technologies appropriate for developing countries (http://web.mit.edu/civenv/html/people/faculty/murcott.html).

Are there synthesis reports or studies? A few arsenic remediation technology papers are 'synthesis' studies: e.g., Ahmed (1999); Chanda and Sinha Ray (1999); Hoque (1999); Murcott (1999). Perhaps the most important synthesis study on arsenic remediation technologies will be the U.N. Synthesis Report on Arsenic, Chapter 6 'Safe Water Technology'. At the time of completion of this paper (September, 2001), this U.N. document is still not available but is expected in October 2001. Recently, Bangladesh University of Engineering and Technology, Dhaka in conjunction with the United Nations University, Tokyo have published an important "synthesis" study: Technologies for Arsenic Removal from Drinking Water (Ahmed et al., 2001).

The World Bank Arsenic Mitigation Water Supply Project Appraisal Document (18252-BD) (May 26, 1998) considered a number of technology options (see Table 1). However, there are no options listed in Table 1 that remove arsenic from tubewell water.

The Bangladesh Arsenic Mitigation Water Supply Project (BAMWSP) with management support of WaterAid Bangladesh and financial backing from the U.K. Department for International Development (DFID) has tested seven tubewell treatment technologies to date (7/2001) of which four were consistently good at removing arsenic without adversely affecting water quality (http://bicn.com/acic/):

- Alcan Enhanced Activated Alumina;
- Sono 3-kalshi;
- BUET Activated Alumina;
- Stevens Institute of Technology Filter.

TABLE 1

Technology options in the World Bank arsenic mitigation appraisal document

Technology option	No. people served	Average incremental cost per person (in taka) (12% discount rate)
Iron removal plant	200	65.42
No. 6 handpump	150	65.76
Tara pump	150	67.64
Ring ('dug') well	200	76.65
Deep tubewell	200	84.83
Rainwater harvesting	20	91.79
Pond filtration	500	104.97

Fig. 1. Picture of a 3-kalshi arsenic purification system. This particular setup has purified 7000 l of contaminated groundwater for drinking. The computerized electrochemical analyzer for arsenic analysis is shown at the background. (Courtesy of Sono Diagnostic Center Environment Initiative, Kushtia, Bangladesh.)

Of the four technologies, the Alcan and the Sono were the two most acceptable to householders, while the BUET and the Stevens Institute of Technology filters were less so. Key factors for social acceptability were cost, ease-of-use and flow rates (Fig. 1).

What about peer-reviewed articles on arsenic remediation technologies? Although there have been a number of conferences to address the arsenic crisis in Bangladesh, many held in Dhaka, Bangladesh, most of these proceedings are unpublished or published in very limited editions. Hence there are many hard-to-get, unpublished papers and 'grey' literature.

METHODOLOGY

The objective of this work has been to develop a database of tubewell treatment arsenic removal technologies appropriate for Bangladesh and other developing countries. The approach has been to use conventional research methods, Internet searches, and personal email correspondence with principal investigators, engineers, various government and non-governmental agencies. The data obtained have been organized into 'Fact Sheets' for each specific technology. The Fact Sheets are catalogued according to the predominant category of treatment process for arsenic removal. There are nine general categories of treatment processes: (1) oxidation; (2) coagulation/co-precipitation; (3) sedimentation; (4) filtration; (5) adsorption; (6) ion exchange; (7) membrane/reverse osmosis; (8) biological; (9) other.

In February 1999, the author presented a preliminary review of appropriate remediation technologies for treating tubewell water in Bangladesh at the 'International Conference on Arsenic in Bangladesh Groundwater,' Wagner College, New York[1]. Twenty-two specific technologies were presented under the general categories of oxidation, coagulation/precipitation, sedimentation, filtration, adsorption, solar distillation and membrane processes. Although these proceedings were unpublished, this first paper provided the foundation on which this more recent work builds.

In this new paper I have now assembled information on 28 additional specific technologies. In June 2000, at the 4th International Arsenic Conference in San Diego, CA, the author presented a 'fact Sheet Template' for organizing the database of arsenic remediation technologies from tubewell water and received feedback from the conference participants on improving this template. The purpose of the template is to provide a means of consistently cataloguing, sharing and updating arsenic remediation technology options. The template is intended to provide essential factual information about each specific arsenic removal technology and to provide published references, principal investigators' names, addresses and emails. The Fact Sheets developed from this template can be found at http://web.mit.edu/civenv/html/people/faculty/murcott.html. Visitors to this site may review all existing, completed or partially completed templates, and those with new information can update information through the "Contact" provided.

Below is a sample Fact Sheet (Table 2), filled out with information on one specific technology, the Sono 3-kalshi.

RESULTS

The complete collection of Fact Sheets provide a database which can be sorted according to key fields. Two key fields or 'technology assessment criteria' are: (1) technology performance; (2) cost.

Technology performance and cost are factual, quantitative elements or technology assessment criteria. 'Social acceptability', as a qualitative criterion, is not a field in the Fact Sheet Template (see Discussion section below for more on 'social acceptability'). Table 3 sorts the Fact Sheet database to highlight and compare performance and cost. (Note: al-

[1] This paper is available at: http://phys4.harvard.edu/~wilson/arsenic_project_main.html

TABLE 2

Sample Fact sheet

Technology name: Sono 3-kalshi
Institution, Agency, Company, NGO:
(1) Sono Diagnostic Center Environment Initiative (SDCEI), Kushtia, Bangladesh (contact Khan et al., 2000a,b; see reference below).
(2) BRAC, Dhaka Community Hospital, Grameen Bank
(3) Department of Public Health Engineering (with Ministry of LGD&C, UNICEF)
(4) NGO Forum (with SDC, DANIDA and CAFOD/EU Partner NGOs)

Technology process(es) (oxidation, passive precipitation/sedimentation with naturally occurring iron; coagulation, sedimentation; filtration; adsorption; ion exchange; membrane processes/reverse osmosis; biological; other): passive precipitation/sedimentation (with naturally occurring iron), filtration, adsorption (zero-valent iron).

Detailed technology description:
A 'kalshi' is the clay water pitcher used for collecting water throughout Bangladesh. A variation on the 3-kalshi system has been a traditional water filtration system in Bangladesh. The 3-kalshi system has recently been adapted as an arsenic removal technology, whereby 3 kalshi are stacked on top of each other and the top 2 kalshi are specially manufactured with a small hole at the bottom. Each kalshi has certain media and functions:
(1) Top kalshi: 3 kg (about 1/6 kalshi volume) iron filings and 2 kg coarse sand. The combined media fills about 1/3 kalshi volume. The rest of the space contains source water for treatment.
(2) Middle kalshi: 2 kg fine sand and 1 kg of wood charcoal of a consistent size, avoiding fine wood ash, which dissolves and produces an undesirable basic water solution. The combined media fills about 1/6 kalshi volume.
(3) Bottom kalshi: collection container. There is no spigot. Filtered water collects in this pot and is directly decanted from this kalshi for use.

Sometimes, a sari cloth is used as an additional filter between the top 2 kalshi.

Equipment needed: 3 kalshi ceramic pots, iron chips, coarse and fine sand, wood charcoal.

Arsenic removal performance (please include initial and final concentration (μg/l), % removal, and specify arsenic species, i.e., As(III), As(V), As(total)): As(III) initial = 73–808 μg/l, As(III) final = 2.2 μg/l below detection limit (1.2 μg/l). As(total) initial = 79–1005 μg/l, As(total) final = 13.6 ± 3 μg/l (Khan et al., 2000b).

Arsenic analytic method, lab or field instrument(s) and detection limit:
Anodic stripping voltammetry (ASV) for trace level As(III) and As(total) and redox potential. Atomic absorption spectrometry with graphite furnace and Zeeman background correction and inductively coupled plasma atomic emission spectrometry to validate measurements of arsenic and measure 24 other metals. Signal detection limit: 1.2 μg/l at 95% confidence level (Khan et al., 2000b).

Other water quality parameters measured (inorganic, organic, microbial):

Laboratory site: Sono Diagnostic Center Environmental Initiative (SDCEI), Courtpara, Kushtia, Bangladesh.
 # Units tested: 10
Unit volume (l) or flow rate (l/day): About 18 l volume per kalshi, flow rate varies from 42 to 148 l/day with new kalshi (Khan et al., 2000b).

Pilot test field site(s): Courtpara, Kushtia, Bangladesh. Charghat Upazilla, Rajshahi (arsenic-free village project, AFV, by Dr. N. Newaz and Prof. K. Rahman) 50
 # Units tested: 50
Unit volume (l) or flow rate (l/day): 20–148 l/day

Full-scale application site(s): Kushtia, Jessore, Rajshahi, Dhaka.
 # Units installed: 11,000 by BRAC supported by UNICEF, 5000 by Dhaka Community Hospital, 2000 by Grameen Bank, 2000 by SDCEI, AFV and other NGOs.
Unit volume (l) or flow rate (l/day): N/A

Contact person (principal investigator or other person(s) responsible for this work): Dr. A.H. Khan, Professor
Address: Department of Chemistry, University of Dhaka, Dhaka, Bangladesh.
Tel: 966-1900 x 4872 **Fax**: **Email**: ahkhan@du.bangla.net **Web Site**:

Contact person (principal investigator or other person(s) responsible for this work): Dr. Abul Hussam, Associate Professor
Address: Chemistry Department, George Mason University, Fairfax, VA 22030.
Tel: (703) 830-5955 **Fax**: **Email:** ahussam@gmu.edu **Web Site**:

Table 2. (continued)

Contact person (principal investigator or other person(s) responsible for this work): Mr. Kazi Nasir Uddin Ahmed, Addi Chief Engineer (Planning), Department of Public Health Engineering (DPHE)
Address: DPHE Bhaban Kakail, Dhaka-1000.
Tel: **Fax**: **Email**: **Web Site**:

Contact person (principal investigator or other person(s) responsible for this work): Mr. S.M.A. Rashid, Exec. Director
Address: NGO Forum, 4/6, Block-E, Lalmatia, Dhaka 1207, Bangladesh.
Tel: 8119597, 8119599 **Fax**: 8117924 **Email**: ngof@bangla.net **Web Site**:

Step-by-step procedure for lab test of this technology:
See Khan et al. (2000b). Lab tests must use real groundwater or groundwater spiked with sodium slats of arsenite or arsenate. The groundwater pH should not change during this spiking.
Step-by-step procedure for field test of this technology:
(1) Assemble 3 kalshis one atop the other. A bamboo or steel frame is generally used to hold the kalshis – see picture.
(2) Boil and wash sand and iron chips to prevent bacterial contamination.
(3) Plug holes in the top 2 kalshis with 100% polyester cloth or similar material.
(4) Fill the top 2 kalshis with appropriate media (see above).
(5) Collect arsenic-contaminated groundwater and fill the top kalshi to capacity (<18 l). Allow water to drain from top to middle to bottom kalshi.
(6) Discard water from the first two kalshi runs before collecting samples for arsenic or other water quality analysis.

Sludge disposal issues:
Total available leaching procedure (TALP)-USEPA, European Union Recommended Sand from 1st and 2nd kalshi: 12 and 16 μg/l As(total) at pH 7; 25 and 15 μg/l As(total) at pH 4. Similar values were obtained for leaching with rain water (unpublished data). Only sand has to be disposed to renew capacity. Actual field test is in progress.

Capacity and costs of arsenic removal technology (please give sludge disposal costs separately below):
Breakthrough capacity: 7000 l at average 500 μg/l As(total) — breakthrough to 60 μg/l As(total), based on laboratory data with real groundwater.
Capital (US $/liter of water treated): about US $5.00 (about 250 takas) for 3 kalshi pitchers and support stand. 0.07 cents/l maximum.
O&M (US $/liter of water treated): 0.07 cents/l maximum

Sludge disposal costs (US $/kg waste generated): N/A

Pros of this technology:
3-kalshi makes use of low-cost, readily available fired unglazed ceramic jugs that are traditionally used for water collection and storage in Bangladesh. Local craftspeople make a variety of sizes and shapes of these jugs.

Cons of this technology:
Breakage of clay pitchers (UNICEF). Availability of cast iron chips. In one unpublished account of a kalshi system pilot test in Bangladesh using 8 l size kalshis, the system was already functioning at 1/2 the original flow rate after 10 days of regular household use due to clogging from flocculation of the naturally occurring iron in the source water. After 10 days, it took an entire day to fill the bottom kalshi.

Published paper(s) describing this specific technology: (please provide electronic or hard copy if possible):
Khan, A.H., Rasul, S.B., Munir, A.K.M., Habibuddowla, M., Alauddin, M., Newaz, S.S. and Hussam, A. 2000. Appraisal of simple arsenic removal method for groundwater of Bangladesh. J. Environ. Sci. Health, A35(7), 1021–1041.

Name of person submitting this information: Susan Murcott
Profession: environmental engineer
Address: 1-138, MIT, Cambridge, MA 02139
Email: murcott@mit.edu
Date submitted: 10/2000

though the template includes an item on cost of sludge disposal, the costs indicated in Table 3 are of the arsenic removal technology itself, not including sludge disposal costs.) Table 3 is a 'work-in-progress'. It draws from a wide range of sources to show current data, some of which are well-established and some of which are preliminary. The reader will note that for certain systems, performance and cost data are not yet available (N/A).

TABLE 3

Performance and cost of technologies for arsenic-contaminated tubewells

	TECHNOLOGY	PERFORMANCE	COST
	OXIDATION		
1	Simple aeration	25%	No cost
2	Solar oxidation (SORAS)	50–80%	Cost of PET plastic bottle + lemon juice
3	UVFeOx (CRC WMPC, Australia) (Wainsberg, 2000)	If As initial 1000 μg/l then As final <50 μg/l	Ferric sulfate = $4.00/yr, Solar troughs = $16, Plastic urns < $16
4	Solar oxidation/reduction (GDR)	94–97%	N/A
	COAGULATION/ CO-PRECIPITATION		
5	(taking advantage of naturally occurring Fe and/or Mn precipitation) (Jones and Uddin, 2000)	Of 17 tubewells tested, some As final <50 μg/l, some As final >50 μg/l. Bacteriological contamination increased substantially in water stored for 12 h in 12 of the 14 tubewells tested.	No cost
	(Khan et al., 2000)	50–70% removal	
6	Coagulation with metal salts	70–90%	$0.06/yr to treat 40 l/day at 15 mg/l $FeCl_3$ dose
7	'Tea-bag' method (Dave, undated) WHO–SEA	80–99%; As final = 30 μg/l	$0.05/packet treats 10 l (cost decrease with mass production)
8	Bucket treatment unit (BTU)	As final:	
	* DPHE/DANIDA reagents	<50 μg/l (81–90%)	$7.00
	* 'Home-made' reagents	35–151 μg/l (78–91%)	$6.00
9	Pilot plant (PHED) – Sujapur P.S. Kaliachak Pilot Plant (Chanda and Sinha Ray, 1999)	As final = 20–30 μg/l	$0.02/1000 l treated
10	Candle filter in plastic bucket (PHED)	As final = 10–140 μg/l depending on coag. dose	$5.33
11	Household sand–gravel G.I. filter (PHED)	As final = 10–140 μg/l depending on coag. dose	
12	Hand-pump-attached arsenic removal system (PHED)	As final = 5–180 μg/l	$426 serves 1200 people, O&M = $102/yr
13	Star household co-precipitation filtration (Stevens Institute of Technology)	As initial = 650 μg/l, As final < 50 μg/l >92%	Bucket system = $9.50, Tablets = $2/yr
14	Oxidation of ferrous iron in acidic conditions (M. Johnson, N.M. State Univ.)	As final = below detection limit	N/A
15	Enhanced iron co-precipitation (Hydro-Solutions and Purification Inc.)	96–99%; As final = 5–27 μg/l	$0.52/1000 l treated
	FILTRATION		
16	'Safi' filter (Jones and Uddin, 2000)	As final > 100 μg/l	$20 + $1.00+/year for replacement candles
	(Safiullah et al., 2000)	100% removal at As initial of 2 mg/l	
17	Filter of indigenous raw materials 1. Coconut coir, shell and husk (developed by Dr. M.A. Hassan of Allergy Env. Research & Skin Care Institute)	N/A	N/A
18	Filter of indigenous raw materials (Bangladesh Center for Science and Industrial Research) (BCSIR)	N/A	US$6.00 (300 Taka)
19	Enviro-main filter (Okanagan University College, B.C. Canada)	N/A	N/A
20	GARNET home-made filter	70–90%	$5.00–$12.00 (Tk 250–600)

TABLE 3. (continued)

	TECHNOLOGY	PERFORMANCE	COST
	ADSORPTION		
21	Activated alumina metal oxide (Apyron Technologies Inc.)	95%; As final < 50 μg/l	<$0.04/80 l assuming 20 l/cap./day. Capital cost $2000 unit (cheaper if produced locally)
22	AMAL hand-pump filter plant with activated alumina (West Bengal Engineering College/Water for People	As max. initial = 480 μg/l, As final < 50 μg/l	Capital cost = $850–$1500; Serves 200–300 families. O&M = $17 per AA regeneration
23	AMAL household-attached activated alumina (West Bengal Engineering College/Water for People)	N/A	N/A
24	Simple filter with zero valent iron (http://www.dainichi-consul.co.jp)	N/A	N/A
25	Iron filings and sand (AsRT – Univ. of Conn.) (Nikolaidis, 2000)	As < 27 μg/l; 90% removal for > 1000 pore volumes of eluent	$0.06/l Capital cost = $900 for pilot unit. Full-scale unit may be $100 (2 columns treating 3.8 l/min)
26	Iron filings in jerry cans (Ramaswami et al., 2000) University of Colorado-Denver	In lab tests > 90%; As final < 50 μg/l; In field tests, arsenic was not successfully removed.	$3.00/yr based on U.S. price of iron filings. Lower in Asia.
27	Sono 3-kalshi (Khan et al., 2000)	99%; As final < 14 μg/l	US$5.00 (250 Taka); O&M max = $0.07/l
28	Design 5 (Univ. of Toronto)	Design parameter As final < 10 μg/l	N/A
29	Ferric oxyhydroxide + settling New Zealand–Bang. Bilateral Dev. Program	As final < 50 μg/l	N/A
30	Ferric hydroxide-coated newspaper pulp (Khair et al., 1999)	As final < 50 μg/l	N/A
31	Granular ferric hydroxide in a packed bed (Driehaus, GDR)	As initial = 100–180 μg/l, As final < 10 μg/l	N/A
32	Solmotex	As initial = 700 μg/l, As final = 10 μg/l	$150/unit; O&M < $150/yr
33	GraMet ferlat – silica and iron adsorbent (GDR)	As initial = 30 μg/l, As final below detection limit of 2 μg/l	< 0.20 DM/m^3 treated
34	Molybdate impregnated chitosan beads (Dambies et al., 2000)	N/A	N/A
35	Laterite	50–90%	N/A
36	Ligand reaction-based ceramic arsenic removal technology (EDA/Luxor)	As initial = 200 μg/l, As final = 1 μg/l; 99.5%	N/A
37	Modified clay absorbent tube (CSIRO, Australia, Kalyani U. + Dhaka U.)	< 50 μg/l	N/A
38	Other adsorbents	N/A	N/A
39	Alcan activated alumina	100%	Capital = $500; O&M = $300/yr
40	BUET activated alumina	As initial = 176–402 μg/l, As final = 2–8 μg/l	Capital = $20.00–$30.00 (1000–1500 Taka); O&M = $7.20–$12.00/yr
	ION EXCHANGE		
41	Tetrahedron (Baltimore, Md.)	As initial = 100–1700 μg/l; 100% removal	$240/unit; O&M = $120/replacement resin, column lasts about 6 months
42	Aquatic treatment systems	N/A	N/A
43	Krudico anion exchange POU filter	95–97%	$0.10/3500 l treated
44	Anion exchange with chloride strong base resins (Clifford et al., 1998)	As final < 2 μg/l	N/A

TABLE 3. (continued)

	TECHNOLOGY	PERFORMANCE	COST
45	Siedko: 2 large cylinders, community-sized system (Harbeusser, Germany)	N/A	$2000/unit
46	Anion nano-engineered sorbents (SANS) Sandia National Lab.	As final = below detection limit	N/A
	MEMBRANE/REVERSE OSMOSIS		
47	HVR membrane distillation POU Purifier (Chand, 2000)	As final = below detection limit (< 3 μg/l)	$1.00/$m^3$ + diesel generator needed
48	Ferric coagulation + microfiltration (Clifford, 1998)	As final < 2 μg/l	N/A
	BIOLOGICAL		
49	Biological filtration (SAUR-U.K.)	As initial = 40–300 μg/l, 98% As reduction	N/A
50	Chemical & biological treatment with duck weed & jute root (Mridha, 2000)	< 50 μg/l	N/A

N/A = not available
References for all entries to Table 3 are available at: http://web.mit.edu/civenv/html/people/faculty/murcott.html

DISCUSSION

Table 3 allows us to compare technologies 'at a glance'. If we assess these technologies according to performance and cost, we see that there are already a number of available technologies which perform well and are low-cost. With such a table, we can screen technologies according to these criteria. But what about 'appropriateness' or 'social acceptability'?

Schumacher (1973) defined an 'appropriate technology' by four propositions.

(1) *Rural areas*: technologies must be created in rural areas where the vast majority of the developing world population lives, and not primarily in metropolitan areas.

(2) *Inexpensive*: technologies must be, on average, cheap enough so that they can be created in large numbers without this calling for an unattainable level of capital formation and imports.

(3) *Simple*: production methods must be relatively simple.

(4) *Local materials and local use*: production should be mainly from local materials and mainly for local use.

The Discussion section of this paper builds on this fourth proposition, i.e., to look at local users. Specifically, it focuses on women's role in appropriate technology projects, based on the author's experience in Nepal, India and Haiti. All over the developing world, including Bangladesh, women collect and carry water for their families, use the water for cooking, cleaning and growing food. Yet women are often not empowered to make important decisions about water. An 'appropriate technology' for arsenic removal from tubewell water in Bangladesh must be socially acceptable to women, as women will be the predominant end-users of arsenic removal technologies in Bangladesh and other developing countries. If an arsenic removal technology is introduced into a village and women do not like it, it will not be used or will be used only reluctantly. I suggest that a proposition or screening criterion — 'social acceptability to women' — be added to

Schumacher's list of four propositions as another defining characteristic of an 'appropriate technology'.

Yet 'social acceptability to women' cannot be presented 'at a glance' as in a Fact Sheet. It can be presented in examples and case studies. Below are three brief examples.

Nepal. In 1998 and 2000, I travelled to Nepal to work with village women, at their request, to come up with a viable and appropriate household water treatment system to handle the widespread problem of microbial contamination of water supplies in Nepal. Three treatment processes were evaluated: manual coagulation with alum, household filtration and solar disinfection. These processes were studied in a laboratory in Kathamandu and at M.I.T. in Cambridge, MA, and were taken to the field to the district of Kavre, where they were demonstrated and tested by women sanitation workers. After a day of hands-on experience, the women said very clearly that they were not interested in manual coagulation with alum. It was too much work, and these women already had too much work. They would not use this process. This was valuable learning, on the basis of which we decided not to pursue alum coagulation further but to concentrate on filtration and solar disinfection, which the women expressed eagerness to implement. (For more information, see video of MIT Nepal Water Project at: http://caes.mit.edu/mvp/html/nepal.html)

India. In the Banda district of Uttar Pradesh, India, the main source of drinking water is from tubewells and women are the carriers and caretakers of that well water. Over the past two decades, as in Bangladesh, international aid agencies have provided grants to government agencies to dig these tubewells. In Banda, the district water engineers of the male-dominated agency responsible for fixing the pumps, the Jal Nigam, had only two mechanics to cover 930 tubewells. Frustrated and without water, illiterate Kohl tribal women decided that "It is better that we learn to fix the pumps ourselves instead of waiting for days for the government to fix our pumps." These women joined together in a voluntary group, Vanagana, the aim of which has been to teach Banda women non-traditional skills including hand-pump mechanics. Its success can be measured by the fact that the Uttar Pradesh State Government is now trying to replicate this project in other areas and has selected it as one project to receive World Bank funds.

Haiti. Gift of Water Inc. (GWI) is a small non-profit organization which has created a household water purification program in Haiti. GWI has set up water committees, established water fees, trained paid water testing technicians on the basis of gender equality and established a woman-run micro-enterprise in Dumay. They have developed a simple, innovative point-of-use water treatment system that has been purchased and distributed to 22,000 people. This system has shown a >95% reduction in waterborne diseases in the areas where these systems are employed. The author has accompanied trained water technicians from circuit to circuit and seen and tested these water treatment systems in household after household. It is typically women who are in these homes using these systems. It was apparent that these women understand and appreciate this technology and use it! They also understand that they share in the economic and social benefits of this project equally with men.

CONCLUSIONS

This paper has pointed out the main features of a new Web site devoted exclusively to low-cost, tubewell water treatment techologies and has presented a summary table of 50 arsenic remediation technologies. This examination leads to a conclusion that a number of arsenic-treatment technology options perform well at a low cost. Are these options socially acceptable to end-users? This is an outstanding question which can only be determined in the field in Bangladesh or other countries with arsenic contaminated drinking water. The end-users of arsenic removal treatment technologies from tubewells in Bangladesh or elsewhere will predominantly be women. Without their full participation, technical

solutions based only on the quantitative screening criteria of performance and cost will likely fail.

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Arsenic Exposure and Health Effects IV
W.R. Chappell, C.O. Abernathy and R.L. Calderon (Editors)
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Surface Properties of an Advanced Adsorbent Developed for Arsenic Removal from Drinking Water

József Hlavay, Klára Polyák

ABSTRACT

A novel type adsorbent was prepared by in-situ precipitation of $Fe(OH)_3$ on the surface of activated Al_2O_3 as a support material. The iron content of the adsorbent was found as 0.313 ± 0.003 m/m% Fe (56.1 mmol/g), its mechanical and chemical stability proved to be appropriate in solutions. The total capacity of the adsorbent was found as 0.115 mmol/g, the pH of zero point of charge, $pH_{zpc} = 6.9 \pm 0.4$. Depending on the pH of the solutions the adsorbent can both be used for binding of anions and cations. If $pH_{eq} < pH_{zpc}$ anions are sorbed on the surface of adsorbent through $\{S–OH_2^+\}$ and {S–OH} groups. The graphical method was used for the determination of pH_{iep} of the surface of the $Al_2O_3/Fe(OH)_3$ adsorbent formed due to the adsorption of arsenic ions. Values of $pH_{iep} = 6.3 \pm 0.3$ for As(III)- and $pH_{iep} = 8.0 \pm 0.2$ for As(V)-ions were found. The amount of surface-charged groups (Q) was about zero within the pH range of 6.5–8.6, due to the practically neutral surface formed on the adsorption of As(V)-ions. At acidic pH (pH = 4.7), $Q = 0.192$ mol/kg was obtained. The adsorption of arsenic ions from dilute solutions (0.1–1.8 mmol/l) was represented by Langmuir-type isotherms. With increasing equilibrium concentration, the monolayer was restructured and new layers were formed.

Keywords: arsenic ions; drinking water; novel type adsorbents; surface properties

INTRODUCTION

The European Commission has recently published a new Directive (98/83/EC) on the quality of water intended for human consumption to achieve a common, high quality standard and to protect public health in its member states. One of the new chemical guideline values is concerned with arsenic, which was lowered from 50 μg/l to 10 μg/l caused by the high human toxicity, namely the high carcinogenic potential. A very recent proposal of the USA Environmental Protection Agency (EPA) put forward an even lower drinking water standard of 5 μg/l. These regulatory proposals are based on a re-evaluation of chronic arsenic toxicity to humans as found in relatively large populations receiving potable water with elevated arsenic levels over several decades.

In Hungary arsenic ions are accompanied by high amounts of ammonium-, Fe-, and Mn-ions, humic acids (about 10–15 mg/l), dissolved gases, and high temperature, >30ºC. Today more than 1.6 million inhabitants in 400 settlements are served with tap water containing As > 10 μg/l. In the water supply of the country the underground waters have an important role. It was found that the maximum frequency of occurrence of arsenic waters containing As > 50 μg/l is generally in the interval of 100–200 m depth. The majority of high arsenic cases is, as far as presently known, due to geogenic occurrence of arsenic minerals in groundwater aquifers, where the compounds dissolve readily and arsenic can be present as reduced As(III) or oxidized As(V) species. This contamination arises from natural leaching of arsenic rocks by the percolating water.

The present state of knowledge and technology is insufficient to solve the arsenic problem by treatment for the different groundwater qualities and arsenic levels found. Conventional techniques have to be modified to upgrade treatment plants or new efficient and environmentally friendly technologies must be developed for economic and safe arsenic removal including the management of the residual solids or liquids. Present experience and investigations demonstrate that the redox status is the most important in view of the arsenic species distribution and the presence of ferrous and manganese, as well as ammonium ions, sulfide, methane and natural organic matter (NOM). The reduced inorganic As(III) is present as non-ionic acid (H_3AsO_3) and to a small extent as monoanionic species ($H_2AsO_3^-$). It has been shown in early studies that As(III) cannot be separated with sufficient efficiency to meet the standard. Oxidative pretreatment is a prerequisite for As(III) in the raw water source. Oxidation can be achieved by chemicals as chlorine, chloramine, chlorine dioxide, ozone, permanganate, Fentons reagent, UV-irradiation or solid oxidants, such as manganese(IV)-oxides.

Oxides, especially those of Si, Al, and Fe, are abundant components of the earth's crust. Hence most of the solid phases in natural waters contain such oxides and hydroxides. Interactions of cations and anions with hydrous oxide surfaces are of importance in natural water systems, in geochemical processes, and in colloid chemistry. A high potential removal process is adsorption on oxide/hydroxide surfaces, either formed in situ (by dosing of coagulants Fe^{3+}/Al^{3+} or by adding ferrous salt and subsequent in-bed oxidation/filtration) or by providing a granulated adsorbent with sufficient capacity and affinity to As(V) (present as $H_2AsO_4^-$ or $HAsO_4^{2-}$). Conventional approaches to the arsenic problems include coagulation/flocculation/filtration with ferric salts, but reliability, safety and residuals treatment are not satisfactory for smaller treatment plants (Jekel, 1994). The use of granular adsorbents with high performance offers the best prospects for a sustainable solution. There is a clear need for inexpensive, but reliable and efficient granular adsorbents for arsenic removal, based on the specific binding to ferric oxide surfaces. Stumm (1987) investigated the thermodynamic behavior of equilibrium interactions on the surface of metal-oxides. Breeuwsma and Lyklema (1973) reported results on the adsorption of ions on hematite in the electrical double layer and samples were prepared by a precipitation technique. Parks and de Bruyn (1962) determined the zero point of charge of

crystalline ferric oxide precipitates. Anderson and Malotky (1979) studied the adsorption of arsenate on different hydrous oxide surfaces as a function of pH_{iep}. In these studies suspensions including solid ferric precipitates have been applied, and the use of solid adsorbents has not been reported yet.

In our work a new adsorbent was developed from environmentally friendly raw materials. The main features of adsorbents were determined as the pH value corresponding to the zero point of charge of the adsorbent (pH_{zpc}), the isoelectric point (pH_{iep}) in equilibrium solutions in the presence of As(III)- and As(V)-ions, the amount of surface charged groups, the surface charge, and the effect of pH on the adsorbed amount of arsenic ions. The adsorbent/solution ratios, the ionic strength, the initial pH of the solutions and arsenic concentrations were changed for the characterization.

EXPERIMENTAL

Preparation of the New Adsorbent

The support of the adsorbent was granulated from Al_2O_3 powder made by the Alum-Earth Plant at Almásfüzito, Hungary (reg. no.: K10). Fractions of the grain size ranging from 0.5 to 1.0 mm were activated at 450ºC over 4 h. The surface of Al_2O_3 was covered by $Fe(OH)_3$ using an in-situ precipitation method. Al_2O_3 was placed in an exsiccator and 10 g/l $FeCl_3$ was added in excess. The air was eliminated by an aspirator pump from the pores. After bubbling, the excess $FeCl_3$ was poured off and $Fe(OH)_3$ was formed on the surface of the granules by NH_4OH solution. The adsorbent was air-dried and it was termed as $Al_2O_3/Fe(OH)_3$. The Fe content of the adsorbent was found as 0.313 ± 0.003 m/m% (56.1 mmol Fe/g), its mechanical and chemical stability proved to be appropriate in solutions. The total capacity of the adsorbent was 0.115 mmol/g.

Methods

The pH value of isoelectric point of $Al_2O_3/Fe(OH)_3$ adsorbent (pH_{iep}) in presence of adsorbate ions was determined by a potentiometric titration. Adsorption experiments consisted of the equilibration of 1.0, 2.5, 5.0, 10.0, and 25.0 g/l adsorbent with a known concentration of anions (1 and 4 mmol/l for As(III)- and As(V)-ions). A series of eight flasks containing the same anion and adsorbent concentration were adjusted to varying pH values by the addition of $HClO_4$ or NaOH. Sodium-perchlorate of 0.1 mol/l and 0.001 mol/l was added to each flask to maintain a swamping concentration of the electrolyte. Solutions were equilibrated in a controlled-temperature shaker at 20 ± 0.5ºC for 48 h (shaker-bath, Kutesz, Hungary). Following incubation, each sample was split into two fractions. One subsample was used for the determination of equilibrium pH. The other subsample was filtered and the residual adsorbate concentration of the supernatant was analyzed. The pH values of equilibrium and initial solutions were measured by a pH meter using a glass electrode (Radelkisz, Hungary). Determination of the As(V)-ion concentration was carried out by spectrophotometric method and the absorption spectra of the arsenate–molibdo–vanadate complex were measured by a Zeiss UV–VIS (Germany) spectrophotometer. Determination of As(III)-ion concentration was performed by coulometric method with an OH 404 type coulometer (Radelkisz, Hungary).

RESULTS AND DISCUSSION

The $Al_2O_3/Fe(OH)_3$ adsorbent had been found to be suitable for removal of arsenic ion content of drinking waters (Hlavay et al., 1984, 1987). Currently the interaction between the adsorbent and arsenic ions was investigated in details. The adsorbent/solution ratio, concentration of As ions, ionic strength and the initial pH value of solutions were changed.

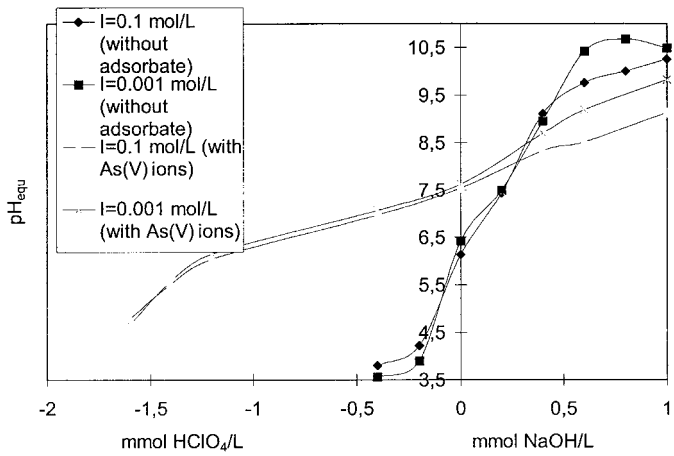

Fig. 1. Determination of pH_{iep} in the presence of arsenic ions. pH values of titration curves of $Al_2O_3/Fe(OH)_3$ adsorbent with and without arsenic ions are plotted against pH_{eq} (1 mmol/l As(V), 1 g/l adsorbent).

Determination of pH Value of Zero Point of Charge (pH_{zpc})

The point of zero proton condition, the pH_{zpc}, corresponds to the pH where the surface is uncharged and is, in the absence of specifically absorbable ions other than H^+ and OH^-, identical with the isoelectric point (pH_{iep}). The zero point of charge of the adsorbent was determined in the presence of adsorbed ions by measurement of the pH value of the isoelectric point (pH_{iep}) of the surface. The pH_{iep} can be determined by potentiometric methods, which is suitable for both colloids and solid materials. Stumm and Morgan (1981) suggested that the pH value corresponding to the intersection point of the titration curves recorded without the presence of adsorbate ions is the pH value of zero point charge (pH_{zpc}). Freshly prepared $Fe(OH)_3/Al_2O_3$ adsorbents in 1.0–25.0 g/l concentrations were used to determine the pH_{zpc} value which was found to be 6.9 ± 0.4.

It is difficult to compare this value with others published earlier, since this advanced adsorbent has not been prepared yet in other laboratories. Stumm and Morgan (1981) published data for different Al and Fe materials, and pH_{zpc} values for α-Al_2O_3, α-$Al(OH)_3$ and γ-AlOOH were found as 9.1, 5.0 and 8.2, respectively, while those of Fe_3O_4, α-FeOOH, γ-Fe_2O_3 and amorphous $Fe(OH)_3$ were obtained as 6.5, 7.8, 6.7 and 8.5, respectively. Points of zero charge for hydrous ferric oxides (HFO) prepared in different methods have been measured by potentiometric acid–base titration (Dzombak and Morel, 1990). Results were in the range of 7.9 to 8.2 with an average value of 8.0. The values are from different investigators who have used different methods and are not necessarily comparable. Goethite and different hematite precipitates were prepared by Atkinson et al. (1967) and pH_{zpc} values of 7.5 ± 0.15 and 8.6–9.3, respectively, were reported. So, the $pH_{zpc} = 6.9 \pm 0.4$ value of this new $Fe(OH)_3/Al_2O_3$ adsorbent is in the lower part of the range as it has been found in other applications for mostly precipitates prepared freshly.

Titration curves of the adsorbent with both As(III)- and As(V)-ions as adsorbate were determined. The potentiometric titration curves were prepared by adding perchlorate or sodium-hydroxide solutions and the volumes were plotted against the pH value of the equilibrium state, pH_{eq} (Fig. 1). In the presence of arsenic ions no intersection points of the titration curves were observed either at 0.1 or 0.001 mol/l ionic strength using 10–25 g adsorbent/l. Recently, it was found that the pH_{iep} of the adsorbent covered by adsorbate can only be determined by the intersection point of the titration curves of solutions with different ionic strengths for samples containing 1–5 g adsorbent/l (Polyák et al., 1995). The pH_{zpc} of adsorbent without adsorbate was related to the titration curve of solutions with

TABLE 1

Determination of pH values of isoelectric point (pH_{iep}) of $Al_2O_3/Fe(OH)_3$ adsorbent using As(III)- and As(V)-ions as adsorbents (graphical method) (ionic strengths 0.1 and 0.001 mol/l)

Adsorbent, g/l	1 mmol As(III)/l		4 mmol As(III)/l	
	0.1 mol/l	0.001 mol/l	0.1 mol/l	0.001 mol/l
1–25	6.68 ± 0.26	5.98 ± 0.36	6.11 ± 0.52	5.52 ± 0.24
Average (pH_{iep}):		6.3 ± 0.3		
Adsorbent, g/l	1 mmol As(V)/l		4 mmol As(V)/l	
	0.1 mol/l	0.001 mol/l	0.1 mol/l	0.001 mol/l
1–25	7.73 ± 0.25	7.92 ± 0.30	8.10 ± 0.48	8.33 ± 0.26
Average (pH_{iep}):		8.0 ± 0.2		

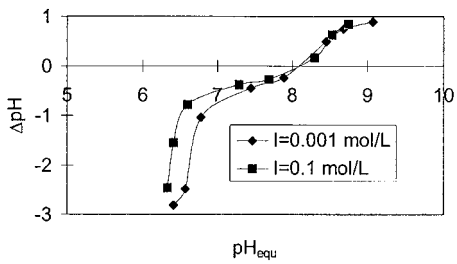

Fig. 2. Determination of pH_{iep} by using ΔpH ($\Delta pH = pH_{in} - pH_{eq}$) as a function of pH_{eq} (1 mmol/l As(V), 5 g/l adsorbent).

the same adsorbent/solution ratio. This graphical method was used for the determination of pH_{iep} of the surface of the $Al_2O_3/Fe(OH)_3$ adsorbent formed due to the adsorption of arsenic ions. An example for 1 mmol/l As(V) and 1 g adsorbent/l is shown in Fig. 1. For both As(III)- and As(V)-ions, the results are summarized in Table 1, and pH_{iep} values of 6.3 ± 0.3 and 8.0 ± 0.2, respectively, were found.

The pH_{iep} values obtained by graphical determination were checked by pH measurements of the solutions. The pH values of the initial and the equilibrium solutions were determined by potentiometric titration and the difference (ΔpH) was calculated. pH_{iep} can be determined as a pH value corresponding to $\Delta pH = 0$, since the charge balance of the adsorbent covered with arsenic ions equals zero (Fig. 2). The pH_{iep} values evaluated by this method are listed in Table 2 for As(III)- and As(V)-ions.

It can be seen that the results of the two models correlate well with each other, with respect to the confidence intervals. In the case of As(III)-ions pH_{iep} (6.3–6.8) was found to be slightly less compared to pH_{zpc} (6.9 ± 0.4), due to the formation of neutral and negatively charged surface compounds. The surface charge became more negative since the H^+ ion dissolution increased the amount of negatively charged groups. The pH_{iep} of solutions with higher ionic strength, with respect to confidence intervals, was equal to the pH_{zpc} due to, mainly, the higher Na^+-ion concentration, which restricts the dissolution of H^+ ions.

Results of pH_{iep} values of $Al_2O_3/Fe(OH)_3$ adsorbent in the presence of As(V)-ions showed that the average pH_{iep} was slightly higher than pH_{zpc}, due to the formation of neutral and positively charged surface compounds resulting from the interaction of the $S–OH_2^+$, S–OH, and $S–O^-$ groups placed on the surface of $Fe(OH)_3/Al_2O_3$ adsorbent. The

TABLE 2

The pH value of isoelectric point of $Al_2O_3/Fe(OH)_3$ adsorbent determined from $\Delta pH–pH_{eq}$ curves using As(III)- and As(V)-ions as adsorbates (ionic strengths 0.1 and 0.001 mol/l)

Adsorbent, g/l	1 mmol As(III)/l		4 mmol As(III)/l	
	0.1 mol/l	0.001 mol/l	0.1 mol/l	0.001 mol/l
1–25	7.10 ± 0.30	6.50 ± 0.40	6.80 ± 0.60	6.80 ± 0.20
Average (pH_{iep}):		6.8 ± 0.2		

Adsorbent, g/l	1 mmol As(V)/l		4 mmol As(V)/l	
	0.1 mol/l	0.001 mol/l	0.1 mol/l	0.001 mol/l
1–25	7.72 ± 0.60	8.35 ± 0.30	8.40 ± 0.20	8.50 ± 0.20
Average (pH_{iep}):		8.2 ± 0.3		

measures of the confidence intervals are attributable to the dependence of surface properties of the adsorbent prepared from different raw materials with various manufacturing processes. These results agree well with the findings of Anderson and Malotky (1979) who investigated arsenate adsorption on amorphous $Al(OH)_3$. The pH_{iep} was determined by the electrophoretical method.

Determination of the Amount of Surface Charged Groups

Interactions taking place on the interface of hydrated oxides/hydroxides play a significant role in the adsorption on the surface of hydrated oxides/hydroxides. For the characterization of these reactions, both the quality and quantity of the surface charges have to be determined. The amount of surface charges can be calculated from the results of potentiometric investigations as:

$$Q = \frac{[H^+]_i - [H^+]_{eq}}{a} \qquad Q = \frac{[OH^-]_i - [OH^-]_{eq}}{a} \tag{1}$$

where: Q is the amount of surface charged groups (mol/kg); $[H^+]_i$ and $[OH^-]_i$ indicate initial concentrations of ions in solutions (mol/l); $[H^+]_{eq}$ and $[OH^-]_{eq}$ are the concentration of equilibrium state solutions (mol/l); a is the amount of adsorbent used, kg/l.

The amount of surface charges formed during the adsorption of As(III)- and As(V)-ions was plotted against the pH_{eq} values. Experimental parameters include 1–25 g adsorbent/l and 1–4 mmol/l arsenic ions, respectively. Only two examples are chosen for illustration in Fig. 3.

Results show that the amount of surface charged groups is about zero within the pH range of 6.5–8.6, due to the practically neutral surfaces formed on the adsorption of As(V)-ions (Fig. 3.). At an acidic pH (pH = 4.7), $Q = 0.192$ mol/kg was obtained. With increasing pH_{eq}, the surface charged groups are again formed in solutions containing 1 mmol As(V) and 1–25 g adsorbent/l. It has also been observed that if the concentration of As(V)-ions was higher (4 mmol/l), the amount of surface charged groups formed on the surface was practically zero at $pH_{eq} > 6.8$, and the surface of adsorbents became neutral up to $pH_{eq} = 9$, the end of the investigated interval. These results are in good agreement with the adsorption of phosphate ions on Al_2O_3 investigated by Stumm and Morgan (1981).

During the adsorption of As(III)-ions (1 and 4 mmol/l on $Al_2O_3/Fe(OH)_3$ adsorbent), in contrast to As(V) ions, a neutral or slightly positive surface was formed around pH_{iep} due to the presence of $S–OH_2^+$ ions (Fig. 3.). The rapidly declining characteristic of curves

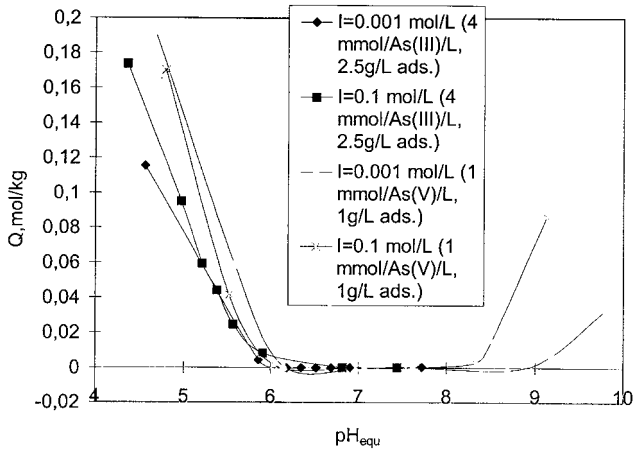

Fig. 3. Amount of surface charged groups as a function of pH_{eq} (1 mmol/l, As(V), 1 g/l adsorbent) and (4 mmol/l, As(III), 2.5 g/l adsorbent).

indicates a reversible reaction. The amounts of surface charged groups formed during the adsorption of As(III)- and As(V)-ions on the surface of $Al_2O_3/Fe(OH)_3$ adsorbent was compared. It was found that the amounts of surface charged groups formed during the adsorption of As(V) ions were significantly higher than those of the adsorption of As(III), due to the possible chemical reactions taking place on the surface of the adsorbent. Within the investigated pH range only As(V)-ions are deprotonated ($H_2AsO_4^-$ and $HAsO_4^{2-}$ are formed), arsenite ions mainly occur as H_3AsO_3. During adsorption, mainly neutralization reactions of As(III)-ions have taken place, while in the course of the adsorption of As(V)-ions, ion exchange reactions are dominant. The amount of surface charged groups formed during the adsorption of As(III)-ions of 1 mmol/l concentration was found to be higher than those in solutions of 4 mmol/l. This phenomenon can be explained by the formation of neutral surface compounds during the adsorption of arsenite ions. The amount of adsorbed As(III)-ions was higher in solutions of 4 mmol/l and 1–25 g adsorbent/l, which led to the creation of less surface charged groups, due to the neutralization reactions of adsorption. For solutions containing As(V)-ions, the amount of surface charged groups was elevated at a concentration of 4 mmol/l, compared to those for 1 mmol/l due mainly to the dominant reaction of ion exchange.

Determination of the Surface Charge of the Adsorbent

Surface charge could be calculated by the following equation:

$$\sigma = \frac{zFQ}{s} \tag{2}$$

where: z is the charge of the surface groups; F is the Faraday constant (96,500 C/mol); Q is the amount of surface charged groups (mol/kg); s is the specific surface of adsorbent (180,000) m^2/kg.

If the pH value of the equilibrium state solution (pH_{eq}) is smaller than pH_{iep}, then $z = +1$ otherwise $z = -1$.

The surface charge was plotted against pH_{eq} for solutions containing As(III)- and As(V)-ions (1 and 4 mmol/l) and the concentration of adsorbents ranged from 1 to 25 g/l. Results of the calculation were fundamentally similar for both arsenic ions. According to this, the surface compounds during the adsorption of As(V)-ions were neutral between the pH range of 6.5 and 8.5 (Fig. 4). If $pH_{eq} < pH_{iep}$ positively charged surface compounds are formed and the surface is covered by $\{S–OH_2^+\}$ groups, and vice versa, if $pH_{eq} > pH_{iep}$, then negatively charged compounds can be found on the surface of the adsorbent due

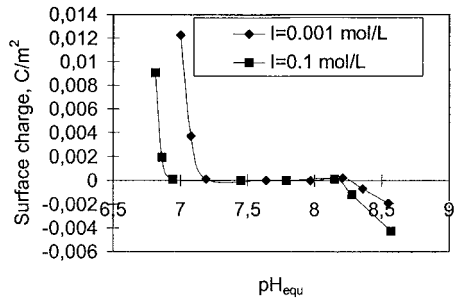

Fig. 4. Surface charge as a function of pH_{eq} (1 mmol/l As(V) 10 g/l adsorbent).

to the adsorption of arsenic ions on the surface of the $Al_2O_3/Fe(OH)_3$ adsorbent. Earlier it was reported that $pH_{zpc} = 6.9 \pm 0.4$ was measured for the adsorbent, and from this a pH value up to pH = 8.2 (value of pH_{iep}) neutral surface was obtained. In this pH range $H_2AsO_4^-$ and $HAsO_4^{2-}$ forms can be expected in drinking waters, so the adsorption of species is effective and the $Al_2O_3/Fe(OH)_3$ adsorbent can be used for purification purposes.

Furthermore, adsorption of arsenic ions from dilute solutions (0.1–1.8 mmol/l) can be represented by Langmuir-type isotherms. The linear form of the Langmuir equation can be written as follows (Posselt et al., 1968):

$$\frac{1}{y} = \frac{1}{\Gamma} + \frac{1}{\Gamma} \cdot \frac{1}{bc} \tag{3}$$

where: y is the quantity adsorbed, mmol/g; c is the equilibrium concentration, mmol/l; Γ is the limiting value for y (monolayer capacity), mmol/g; b is a constant relating to the energy of sorption.

Adsorption isotherms are presented in Fig. 5. Only results for As(III)-ions are shown; the isotherms are similar for As(V) species. The initial fractions of the isotherms (0.1–0.4 mmol/l) have been linearized, and the results are summarized in Table 3. As it can be seen from the values of r (linear regression coefficient of Langmuir curves) and Γ, the adsorption can properly be described by the Langmuir equation for the smaller metal concentrations (<0.4 mmol/l). Giles et al. (1960) found that if the adsorption process followed the L-type curves, the adsorbed molecules were most likely adsorbed flat. The adsorbate is in an intermolecular relationship with the surface and competes with the water for the active adsorption centers of the surface.

The adsorption isotherms show different features (Fig. 5). After the initial Langmuir-type curve, a plateau can be seen, and a further increase indicates the completion of the monolayer. The plateau must represent a first-degree saturation of the surface, i.e., the

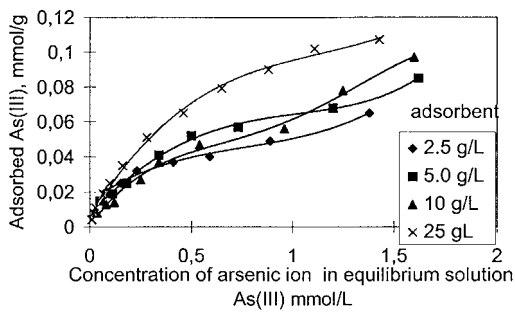

Fig. 5. Adsorption isotherms for As(III)-ions at pH = 6.62–6.74.

TABLE 3

Parameters of Langmuir equation for different adsorbate/adsorbent ratios

Adsorbent (g/l)	r	Γ (mmol/g)	b
As(III), pH = 6.6–6.7			
2.5	0.991	0.052	6.39
5	0.997	0.101	1.92
10	0.999	0.082	2.4
25	0.996	0.102	3.3
As(V), pH = 7.15–7.2			
2.5	0.991	0.489	0.92
5	0.958	0.192	16.28
10	0.999	0.179	0.8
25	0.945	0.212	0.82

r = regression coefficient of the Langmuir linear curves; Γ = monolayer capacity, mmol/g; b = a constant relating to the energy of sorption.

condition at which all possible sites on the original surface are filled and further adsorption can take place only on new surfaces. This degree of saturation may be called the formation of the complete monolayer, but this does not necessarily imply that this monolayer is a closely packed layer of single molecules or ions (Giles et al., 1960). Using the $Al_2O_3/Fe(OH)_3$ adsorbent, adsorption of arsenic species is taking place by chemical reaction through active groups of O^- and OH^-. With increasing equilibrium concentration, the monolayer was restructured and new layers were formed.

CONCLUSION

A novel type adsorbent was prepared by an in-situ precipitation of $Fe(OH)_3$ on the surface of the activated Al_2O_3 as a support. The Fe content of the adsorbent was found as 0.313 ± 0.003 m/m% (56.1 mmol/g), its mechanical and chemical stability proved to be appropriate in solutions. The total capacity of the adsorbent was 0.115 mmol/g, the pH of zero point of charge, $pH_{zpc} = 6.9 \pm 0.4$. Depending on the pH of solutions the adsorbent can both be used for binding of anions and cations. If $pH_{eq} < pH_{zpc}$, anions are sorbed on the surface of adsorbent through $\{S–OH_2^+\}$ and {S–OH} groups.

ACKNOWLEDGEMENTS

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Critical Evaluation of a Simple Arsenic Removal Method for Groundwater of Bangladesh

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ABSTRACT

A simple three-pitcher (locally known as '3-Kolshi') water filtration system made from locally available materials was tested for its efficacy in removing arsenic, other trace metals and anions from the groundwater of Bangladesh. In a 3-Kolshi system, the first Kolshi has cast iron turnings and sand, the second Kolshi has wood charcoal and sand as the active ingredients. About 6000 l of groundwater containing 80–1900 μg/l of arsenic was filtered. The filtered water contained 12 ± 10 μg/l As(total), no detectable As(III), and significantly reduced major, minor and trace metals. The filtration process was monitored for a year by measuring As(total), As(III), 23 other metals, 9 anions, pH, conductivity, temperature and flow rate. The complete conversion of Fe^0 to nonmagnetic hydrous ferric oxide as the most active component for arsenic removal is indicative of an oxidizing environment sustained by continuous diffusion of air through the porous Kolshi. All parameters indicate that the water quality meets and exceeds USEPA, WHO and Bangladesh standards. Leaching experiments of solid wastes showed no significant change in water quality. At its present capacity, five people can use the system for about five months at 50 l/day consumption. Regeneration of the system to its original efficiency can be obtained by changing the sand in the Kolshi. These low cost units (ca. US$ 5.0–6.0) are used by people in the arsenic-affected areas of Bangladesh in large numbers.

Keywords: 3-Kolshi; zero valent iron; arsenic removal; groundwater; Bangladesh

INTRODUCTION

Recent measurements show that in many parts of the Ganges and Brahmaputra basin more than 60% of the shallow and deep tubewell water contains arsenic above the WHO guideline value of 10 μg/l (microgram per liter or parts per billion, ppb) and more than 30% of the tubewells contains arsenic above the maximum contamination level (MCL) of 50 μg/l (Chatterjee et al., 1995; Das et al., 1995). It is estimated that of the 125 million people of Bangladesh, between 35 and 77 million are drinking groundwater containing more than 50 μg/l MCL. The contaminated water is widely hand pumped from a depth of 30–200 feet and there are about 2.5 million tube wells according to WHO estimates, although the unofficial estimate is about 10 million (Smith et al., 2000). The prolonged drinking of this water has caused a serious health hazard in the form of hyperkeratosis on the palms and feet (Choudhury et al., 1998). Long-term exposure to low concentrations of arsenic has been reported to cause cancer of bladder, skin and other internal organs (International Agency for Research on Cancer, 1980).

The health hazard caused by drinking arsenic-laced water can be arrested by drinking arsenic-free water because the biological half-life of arsenic appears to be between ten hours and four days (Tam et al., 1979; Goyer, 1996; National Research Council, 2000). There are no known cures for arsenicosis. Since 97% of the population depend on groundwater, provision of safe drinking and cooking water warrants immediate development of water purification systems appropriate for rural Bangladesh. This work is motivated by the urgency of the current crisis and the need for a simple and low-cost technique for the removal of arsenic from the groundwater of Bangladesh. Considering the vulnerability of the vast population to adverse health effects of arsenic, developing an appropriate, affordable household filtration system is a major step in combating the current calamity. Recently, we have reported such a method and the physicochemical basis for the purification of arsenic-contaminated groundwater (Khan et al., 2000a,b). The arsenic filtration system assembled by using locally available materials has been widely accepted and promoted by various non-governmental organizations in the arsenic-affected areas of Bangladesh in large numbers (est. 20,000 units are in use). This work is a one year critical evaluation of the 3-Kolshi filtration system based on measurements of As(III), As(total), 23 major, minor and trace metals, 9 anions, pH, conductivity and flow rate.

METHODOLOGY

Filtration System Setup

The filtration system described here and in our earlier report (Khan et al., 2000a,b) is based on fired, unglazed clay pitchers (hereafter called by its local Bengali name 'Kolshi') used by more than 80% of the population as a reservoir for drinking and cooking water. Fig. 1a shows the filtration system used in this study. In a three-Kolshi (3-Kolshi) filtration system, the Kolshis (top, middle, and bottom) are placed on top of each other in a steel or bamboo frame for ease of maintenance. Each Kolshi has a volume of about 18 l. The top and middle Kolshi has small holes (0.5–1.0 cm diameter) and covered with pieces of synthetic (polyester) clothes slightly pushed inside the holes. Small brickette pieces, about 0.5 kg each (grade A red bricks, 2–3 cm pieces), were spread on the clothes. The middle Kolshi was then filled with 2 kg sand (from the local Garai river), 1 kg wood charcoal (ca. 1 cm pieces from cooking wood) and 2 kg brickette pieces. The top Kolshi has 3 kg of cast iron turnings (from a local machine shop or iron works) placed uniformly on the brickettes and 2 kg of sand on top of the iron turnings. Tubewell water is poured slowly on the top Kolshi and collected at the bottom Kolshi. The filtration system was used after discarding initial 3–4 batches (ca. 10 l each) of water. Experience shows that covering the middle and bottom Kolshis with small pieces of synthetic clothes placed on

Fig. 1. A 3-Kolshi filtration setup. This particular setup was used to filter 6000 l of arsenic-contaminated groundwater. The computerized electrochemical analyzer for the measurement of arsenic is shown in the background.

perforated covers (available from the same Kolshi makers), can prevent accidental leakage and dusts. Users are advised to follow general precautions such as the placement of the filtration unit in a secure place, keeping the top opening of the Kolshis always covered with clean clothes and practice hygiene rules in handling drinking and cooking water. The water was collected for analysis by decanting into prewashed HDP (high-density polyethylene) sample collection bottles and analyzed immediately for As(III) and As(total) by the computerized electrochemical analyzer.

Analytical Methods and Procedure

Details of analytical methods and procedure for the measurement of As(III), As(V), Fe(soluble), pH, Eh, and conductivity were described earlier (Khan et al., 2000a,b). It should be noted that water samples were collected before and after filtration in acid pre-washed HDP bottles and promptly analyzed for As(III), As(total), and Fe(total) without further filtration. This ensures analytical data from actual drinking water. Samples for ICPAES (inductively coupled plasma atomic emission spectroscopy) analysis of 24 metals were preserved in 250 ml HDP bottles acidified with 1.0 ml concentrated HCl (AnalaR) without a headspace. Samples for anion analysis were preserved without acid.

Selective measurements of As(III) were performed by a computer-controlled electrochemical analyzer (Model HQ-2040, Advanced Analytics, Virginia, USA) following a modified EPA method 7063: arsenic in aqueous samples and extracts by anodic stripping voltammetry (ASV) (Pyles and Miller, 1999). As(total) was measured by the same technique after chemical reduction of As(V) to As(III). Detail of procedure, analytical merits and method validation are described elsewhere (Davis et al., 1978; Sun et al., 1997; Rasul et al., 1999a; Khan et al., 2000a,b). A continuous flow hydride generation atomic absorption spectrometer (Buck Scientific, Model 210 VGP at Intronics Technology Center, Dhaka, Bangladesh), a Perkin-Elmer model 5100 Zeeman-effect atomic absorption spectrometer with a graphite furnace (AASGF-Z), and model A-60 autosampler (at GMU, USA) were used for regular measurement checkup and cross validation of field measurements. A 24-trace metal profile of water samples before and after filtration was measured by a direct reading Echelle ICPAES with radial and axial view (Leeman Labs, NH, USA). The anion analysis of preserved water samples was performed by a Lachat QuickChem Model 6000 Ion Chromatograph (Zellweger Analytics, WI, USA). Total alkalinity, and the concentrations of $H_2CO_3(aq)$, CO_3^{2-} and HCO_3^- were determined from acid base microtitration of groundwater.

RESULTS AND DISCUSSION

Nature of Groundwater

In Bangladesh, the groundwater chemistry is not very well known. The development of any filtration system requires a relatively clear understanding of the water being filtered and its composition. The present development work was carried out in Kushtia Sadar with a population of 400,000 in 316 km^2 (Statistical Pocketbook of Bangladesh, 1994). It is now known that over 40% of the groundwater in Kushtia is contaminated with more than 50 $\mu g/l$ As(total) of which 43–98% is present in the more toxic form of As(III) (Rasul et al., 1999b). This is possibly one of the most toxic groundwaters for direct consumption. Table 1 shows the composition of six replicates of anoxic groundwater obtained from two tubewells used for the present filtration studies. The elemental composition shown as the concentration range and the anion compositions are the average of three replicates. Except for the total arsenic concentrations (80–1900 $\mu g/l$) the water from these wells are representative of the drinking water from this area. Typically, the groundwater is rich in soluble iron that upon leaving for 2–6 h in an oxic environment turns turbid and the formation of a brownish colloidal hydrous ferric oxide is observed. Spectrophotometric measurement of turbidity at 450 nm shows a 30% decrease in transmittance from a clear solution in 4 h (Rasul et al., 2000. The water is also high in calcium and magnesium and thus can be regarded as hard water. The choice of these two tubewells for the present study was prompted by the high concentrations of arsenic and other minerals that clog many commercial filters and constitute a stringent test for the long-term effectiveness of the 3-Kolshi filtration system. Table 1 also lists the composition of the 3-Kolshi filtered tubewell water as discussed later.

The concentrations of anions, NO_2^-, NO_3^-, SO_4^{2-}, Cl^-, F^-, Br^-, and PO_4^{3-}, listed in Table 1, were obtained by ion chromatography of water preserved without acid. The concentration of these anions may be underestimated due to precipitation which has affected the multivalent anions (SO_4^{2-} and PO_4^{3-}) more than the monovalent anions (Cl^-, F^-, NO_3^-, and NO_2^-). The concentrations of CO_3^{2-}, $H_2CO_3(aq)$, and HCO_3^- were based on acid–base titration of fresh groundwater (Snoeyink and Jenkins, 1980). To understand the speciation of the groundwater, a computational geochemical model, MINEQL+ (Schecher and McAvoy, 1998) was used with the inputs as shown in Table 1. The speciation model shows Ca^{2+}, Fe^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Sr^{2+}, Zn^{2+}, Cl^-, CO_3^{2-}, F^-, SO_4^{2-}, NO_3^-, and

TABLE 1

Composition of typical groundwater from two tubewells and the 3-Kolshi filtered tubewell water (conversion factor: 1 mg/l = 1000 μg/l)

Species and parameters	Groundwater (mg/l)	3-Kolshi water (mg/l)
Aluminum, Al	<0.015–0.052	<0.015–0.033
Antimony, Sb	<0.013–0.017	<0.013
Arsenic, As(total)	0.08–1.900	0.012 ± 0.010
As(III)	0.073–1.170	<0.002
Barium, Ba	0.161–0.170	0.05–0.063
Beryllium, Be	<0.001	<0.001
Cadmium, Cd	<0.001	<0.001
Calcium, Ca	111–117	56–65
Chromium, Cr	<0.002	<0.002
Cobalt, Co	<0.002	<0.002
Copper, Cu	0.004–0.009	0.005
Iron, Fe	6.77–7.19	0.010–0.49
Lead, Pb	<0.004–0.005	<0.004–0.006
Magnesium, Mg	21.4–23.1	19–27
Manganese, Mn	0.69–0.74	<0.002
Molybdenum, Mo	0.001–0.003	0.001–0.003
Nickel, Ni	<0.002	<0.002
Potassium, K	1.88–2.45	3.4–16.7
Selenium, Se	<0.012	<0.012
Silver, Ag	<0.002	<0.002
Sodium, Na	18.2–20.4	20.1–92.4
Strontium, Sr	0.280–0.297	0.18–0.37
Thallium, Tl	<0.067	<0.067
Tin, Sn	<0.002–0.01	<0.002–0.01
Vanadium, V	<0.001	0.004–0.009
Zinc, Zn	0.012–0.021	0.011
pH	6.9–7.2	7.7±0.2
Conductivity, μS/cm	570 ± 46	365 ± 66
Temperature, °C	27–29	27–30
NO_2^-	0.469	<ic
NO_3^-	0.980	0.823
SO_4^{2-}	0.424	2.43
Cl^-	3.273	5.9–110
Br^-	<ic	0.028
F^-	0.27	0.47
PO_4^{3-}	0.023	0.068
CO_3^{2-}	0.29	1.47
H_2CO_3(aq)	148	18.3
HCO_3^-	560	432

<ic = below the detection limit of the ion chromatographic system.

NO_2^- as the major *components* and H_3AsO_3(aq), $HAsO_4^{2-}$, $H_2AsO_4^-$, $CaHCO_3^+$, $CaCO_3$(aq), $MgCO_3$(aq), $MgHCO_3^+$, $MnHCO_3^+$, H_2CO_3(aq), and HCO_3^-, as the major *species* (concentration $>10^{-6}$ M) under anoxic conditions. The model also predicts that the concentration of Fe(total), Mn(total), Al, Ca, Ba, and CO_3^{2-} exceeds their solubility product limits for minerals — hematite (α-Fe_2O_3), bixbyite ($Mn(OH)_3$), diaspore ($Al(OH)_3$, $2H_2O$), calcite ($CaCO_3$) and dolomite ($CaMg(CO_3)_2$) — and thus precipitate out under oxic conditions (Rasul et al., 2000). Similar oxic conditions prevail in the 3-Kolshi filtration system.

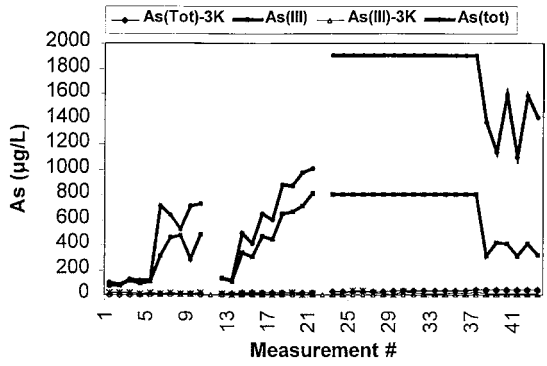

Fig. 2. Arsenic removal efficiency of a 3-Kolshi filtration system. The 3-Kolshi filtered data (-3K) are shown on the abscissa. See text for details.

Effectiveness of Arsenic Filtration System

Fig. 2 shows the effectiveness of the 3-Kolshi filtration system for the removal of As(III) and As(total) from 6000 l of groundwater. The numbers indicate measurement at regular intervals except for the breaks at 10th and 22nd measurements. The first break was due to the change in the filter plug in the middle Kolshi and the second break was caused by the breakage of the top Kolshi, which was filled with new sand. These changes, however, did not decrease the arsenic removal efficiency as shown in Fig. 2. Measurements 1–5 were due to the filtration of original groundwater containing 80–100 μg/l As(total) and 75 μg/l As(III). The filtered water contained 17 μg/l As(total) and <2 μg/l As(III). At this point the same groundwater was spiked with arsenite (As(III): As_2O_3 dissolved in dilute NaOH and neutralized) and arsenate (As(V) in Na_2HAsO_4) to increase the concentrations of As(total) and As(III) at indicted levels. In order to facilitate and observe the 50 μg/l breakthrough, 6 l groundwater spiked with 10,000 μg/l of arsenate was filtered. The resulting effluent contained arsenic at a level of 22 μg/l As(total) (not shown in Fig. 2). Clearly, no breakthrough appeared. The total volume of water filtered from this tubewell was ca. 4850 l. Measurements 24–38 were made with groundwater from another tubewell (Harishankarpur, Kushtia Sadar) which was known to have a maximum of 1900 μg/l As(total) and 800 μg/l As(III). Clinically identified arsenical keratosis patients are reported in this location. Measurements 24–38 were carried out in quick successions so as to maintain the high concentration of arsenic species. Concentrations of both As(total) and As(III) were found to fluctuate in this location when water was collected at long irregular intervals as shown by the remaining data. The total volume of water filtered from this tubewell was ca. 1000 l. In all cases the filtered water contained 12 ± 10 μg/l As(total), which is close to the WHO guideline value of 10 μg/l and no detectable As(III), irrespective of the input levels of As(total) and spiking.

In a continuation with our previous work, measurements of total soluble Fe, pH, conductivity and flow rate were made intermittently to judge the quality of water. These results and the elemental composition are summarized in Table 1. We also note that the filtered water remained clear for months in clear plastic water bottles. Table 1 shows that the concentrations of Fe decreased significantly and sometimes were below the detection limit of ICPAES. The concentration of Mn also decreased to less than 2 μg/l. Therefore, these two metals are quantitatively removed by the filtration system as their hydroxide precipitates. While the concentration of Ca and Ba are decreased by half, the concentration of Mg did not change. The concentration of Na also did not change (except for one high reading) while the concentration of K increased slightly. This could be an indication of a very weak dissolution or ion exchange dissolution of sand minerals (Langmuir, 1997). A slight increase in V concentration in the filtered water is an indication of the presence of

V in the cast-iron turnings. Zn concentration decreased by half. The concentrations of all other trace metals did not change or remained below the detection limit of ICPAES. The pH of the filtered water was increased by one unit, possibly a result of decarbonation. This is also evident from the decrease in bicarbonate concentration. A 36% decrease in solution conductivity is indicative of substantial removal of ionic components from solution. Anion concentrations in the filtered water are generally low (except for one high reading for Cl^-) and had no consequence either on the filtration system or on speciation. Water chemistry shown in Table 1 is in agreement with our previous findings at the early stage of filtration system development, which indicates the consistency and reproducibility of the 3-Kolshi filtration system. Except for occasional variations in non-toxic species (Na and Fe), and a change in flow rate, the 3-Kolshi system performed well.

The 3-Kolshi system described here was used to filter about 6000 l of groundwater after which the effluent arsenic increased to about 60 μg/l As(total) without a clear rise in concentration as would be expected from classical breakthrough experiments (not shown in Fig. 2). In some cases, the system may not function adequately due to clogging of the outlets and overloading of sand with fine hydrous ferric oxide (HFO: Fe_2O_3, 2–3 H_2O) precipitates. Experience shows that gentle poking of the outlet filters with a fine sewing needle can restore the flow rate. Regeneration of the system to its original efficiency can be obtained by changing the sand in the Kolshi. The drinking water production rate (6–7 l/h) is adequate for drinking and cooking purpose of a family of 4–5 persons. At this capacity, five people can use the system for five months at 50 l/day consumption. Further improvements in flow rate by redesigning the outlet system with a free flow junction are in effect

One of the present concerns with any filtration system is the disposal of the solid waste produced. We estimate that the concentration of As(total) accumulated in sand and HFO is 350 mg As/kg of solid. Since the filtration system requires no chemical regeneration steps (e.g., in regeneration of activated alumina column for arsenic mitigation) the wastes produced by Kolshi are self contained. Preliminary experiments involving the Dutch Total Available Leaching Procedure (TALP) of sand and mixed waste from the 3-Kolshi show 15 μg/l and 25 μg/l of As(total) at pH 7 and pH 4, respectively (ASTM, 1992; Price, 1997). Similar results (<16 μg/l by ICPAES) were obtained for leaching with rain water instead of deionized water. ICPAES data of the same solutions show no increase in concentrations of other metals compared to that of the filtered water (Hussam et al., 2000).

Role of Iron in Arsenic Removal

Iron is present in the filtration system as zero valent iron, Fe^0, in the top Kolshi and in groundwater mostly as soluble Fe(II) species. Since the filtered water is nearly free from iron, the oxidation products of zero valent iron and the oxidation of Fe(II) species to HFO (Dzombak and Morel, 1990) are quantitatively retained in the top and middle Kolshi. Previously, we reasoned that arsenate was quantitatively removed by compound formation or adsorption on HFO (Khan et al., 2000a,b). In both cases, the excess arsenic removal capacity increases linearly after each Kolshi of filtration. This is due to the accumulation of HFO formed from freshly available soluble iron in groundwater. We calculate that the excess capacity due to compound formation ($FeAsO_4$(s)) is 1.2 mg As/mg Fe and for adsorption, 0.037 mg As/mg Fe. Because the excess capacity is a measure of under-used efficiency of arsenic removal, a very large volume of groundwater can be filtered before overloading the system.

The role of various forms of zero valent iron was studied by several groups as a means to remediate environmental contaminants such as arsenic, dissolved heavy metals and chlorinated hydrocarbons (Roberts et al., 1996; Driehaus et al., 1998; Shokes and Moller, 1999; Ponder et al., 1999; Lackovic et al., 2000). Studies by others were aimed at mitigating EPA super-fund sites containing high concentrations (mg/l) of arsenic from industrial

wastes and artificial waste water (Nikolaidis and Dobb, 1998). To assess the role of Fe^0, two experiments were performed. The experiments involve one filtration column made of 50 ml plastic hypodermic syringe filled with 25.0 g sand and the other filled with 15.0 g cast-iron turnings. About 500 ml of groundwater spiked with 500 μg/l As(total) was filtered through these columns. The effluents from the sand column had 250 μg/l As(total) and that of 20 μg/l As(total) from the column containing cast iron turnings. Therefore, in comparison to sand, the Fe^0 has a significant capacity to remove arsenic even when no visible HFO formation was observed.

At the end of the present 3-Kolshi experiment, it was found that Fe^0 in the top Kolshi was turned into a solid cemented brownish iron oxide with visible pores throughout the mass. The solid mass was completely non-magnetic. It is clear that extensive oxidation of Fe^0 took place inside the Kolshi, which was sustained by a continuous diffusion of air and water vapor through the porous ceramic Kolshi. There exist various mechanistic pathways for the chemical and physical transformation of Fe^0 surface (Raven et al., 1998; Ponder et al., 1999; Lackovic et al., 2000). It appears, however, that HFO formation by sustained oxidation of Fe^0 and Fe(II), formation of a positively charged double-layer in presence of excess Fe(II)/Fe(III) on the HFO surface and subsequent specific adsorption of arsenate ($H_2AsO_4^-$ and $HAsO_4^{2-}$) are the primary mechanisms for the removal of arsenic (; Gulledge and O'Connor, 1973; Pierce and Moore, 1982; Dzombak and Morel, 1990). Moreover, in the presence of a high concentration of Ca^{2+} the positive charge density of the HFO colloids can increase and thus enhances adsorption of arsenate: $H_2AsO_4^-$, $HAsO_4^{2-}$ and AsO_4^{3-} (Wilkie and Herring, 1986). It is also noteworthy that the oxidizing environment has removed all traces of Mn(II) and NO_2^- from the groundwater.

Quality of Filtered Water

Table 2 shows the inorganic quality parameters of filtered water in comparison to the requirements set by USEPA, WHO and Bangladesh guidelines. Clearly, the water obtained from the 3-Kolshi filtration system meets and exceeds international drinking water standards. Recently, the 3-Kolshi filtration system underwent a series of tests commissioned by the technical advisory group of the Bangladesh Arsenic Mitigation Water Supply Project (BAMWSP) and was found to perform consistently well throughout, passing the 50 μg/l threshold at all wells where it was tested (Rapid Assessment, 2001). The report shows average arsenic concentrations of 9–16 μg/l As(total) in the filtered water and an average flow rate of 5.0 l/h irrespective of water chemistry from four distinctly different locations (Sitakundu, Hajiganj, Iswardi, and Kalaroa) in Bangladesh. These results are consistent with our present and previous findings.

The water quality parameters do not include information on the presence of pathogenic bacteria because groundwater is presumed to be free from such bacterial load. Many such 3-Kolshi systems are in regular use throughout Bangladesh including SDCEI and the authors' home. The 3-Kolshi water is clear, light tasting and free from the metallic taste of groundwater generally associated with high iron and calcium.

CONCLUSIONS

The simple arsenic filtration system presented here is promising because it is based on a clear physicochemical principle, uses locally available materials without adding chemicals. A possible scale-up of the system to a community-based rural and urban pure drinking water supply has been planned. We believe that the 3-Kolshi system can be very effective for filtration of toxic groundwater in Bangladesh and in many parts of the world where clay pitchers are used for preserving drinking water.

TABLE 2

Drinking water inorganic quality parameters: comparison of 3-Kolshi water with those of USEPA, World Health Organization (WHO) and Bangladesh Standards [a]

Constituent	USEPA (MCL)	WHO Guideline	Bangladesh Guideline	3-Kolshi water
Arsenic(total), mg/l	0.05	0.01	0.05	0.012 ± 0.010
Iron(total), mg/l	0.3	0.3	0.3 (1.0)	0.010–0.49
Sodium, mg/l		200		20.1–92.4
Calcium, mg/l			75 (200)	59.1 ± 7.5
Copper, mg/l	1.3	1.0–2.0	1.5	0.005
Manganese, mg/l	0.05	0.1–0.5	0.1 (0.5)	<0.001
Zinc, mg/l	5	3.0	5 (15)	0.011
Aluminum, mg/l	0.05–0.2	0.2	0.1 (0.2)	<0.015–0.033
Lead, mg/l	0.015	0.01	0.10	<0.004–0.006
Chromium, mg/l	0.1	0.05	0.05	<0.002
Cadmium, mg/l	0.005	0.003	0.01	<0.001
Barium, mg/l	2.0	0.7	1.0	0.063 ± 0.01
Antimony, mg/l	0.006	0.005		<0.013
Molybdenum, mg/l		0.07		0.003
Nickel, mg/l	0.1	0.02		<0.002
Selenium, mg/l	0.050	0.01		<0.012
Silver, mg/l	0.1			<0.002
pH	6.5–8.5	6.5–8.5	6.5–8.5	7.7 ± 0.2
Sulfate, mg/l	250 [b]		100	2.43
Fluoride, mg/l	4.0		1.0	0.47
Chloride, mg/l	250	250	200 (600)	5.9–110
Bromide, mg/l				0.028
Nitrate, mg/l	10		10	0.823
Nitrite, mg/l	1			0.001 (<ic)
Phosphate, mg/l			6	0.068
Total dissolved solids (TDS), mg/l	500	1000	500 (1500)	208

[a] Bangladesh standard values are given as maximum desirable concentration with maximum permissible concentration in parentheses. TDS for 3-Kalshi was calculated from the conductivity data excluding silica present in the filtered water. The symbol < indicates below the detection limits of ICPAES. Anions SO_4^{2-}, F^-, Cl^-, Br^-, NO_3^-, and PO_4^{3-} were measured by ion chromatography of samples collected at 5000 l of filtration. <ic = below the detection limit of ion chromatography.

[b] Secondary maximum contaminant level.

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Innovative Technologies for Remediation of Arsenic in Soil and Groundwater

James C. Redwine

ABSTRACT

The Southern Company (SC) and the Electric Power Research Institute (EPRI) have developed several technologies to clean arsenic from soil and groundwater, including: solidification/stabilization (S/S) with select additives; soil flushing; combined iron coprecipitation and ceramic membrane filtration; electroremediation or electrokinetics; in situ chemical fixation; and natural attenuation modeling. Two sites were treated to closure with the S/S method. At another site, soil flushing and combined iron coprecipitation/ceramic membrane filtration reduced an arsenic plume by about 73% in 6 months; the plume stabilized at about 20% of its historical maximum after only 18 months of treatment. The iron coprecipitation/ceramic membrane water treatment process reduced influent arsenic concentrations of about 0.5 to 1 mg/l to less than the drinking water standard (0.05 mg/l) or lower (<0.005 mg/l). Based on successful treatability studies, an electroremediation field demonstration is under way. Phytoremediation and in situ chemical fixation treatability studies also showed positive results. The SC used EPRI's MYGRT 3.0 model to simulate existing plumes at three sites, and to predict arsenic reduction in groundwater after source abatement. Source removal was performed at one of the sites, and groundwater monitoring data to date confirm MYGRT's prediction.

Keywords: arsenic; remediation; iron coprecipitation; electrokinetics; phytoremediation; natural attenuation

INTRODUCTION

Arsenic is a widespread contaminant, arising from a variety of activities including smelting of ores, and the manufacture and use of pesticides and herbicides. As a result, soil and/or groundwater contamination occurs at multiple sites. In the late 1980s and early 1990s, the SC assessed and prioritized multiple sites for remediation. At that time, few successful case histories of arsenic remediation existed in the literature. In 1993, the SC, cofunded by the Electric Power Research Institute (EPRI), began a program of developing technology for arsenic clean-up in soil and groundwater. Since conditions vary from site to site, no single remedial technology is applicable to all sites. This paper describes the technologies developed to date, successful case histories, and studies under way.

SOLIDIFICATION/STABILIZATION

Solidification/stabilization (S/S) describes the technique of solidifying a contaminated soil or waste material (e.g., a sludge), to immobilize the contaminant both chemically and physically, and to reduce the leaching potential to groundwater. Solidification refers to the addition of a binder to produce a solid. Stabilization refers to the addition of a chemical agent to convert the soil or waste material to a more chemically stable form (Miller, 1996). Some additives, such as portland cement, produce both physical and chemical changes. In this project, treatability studies were performed on arsenic-contaminated soils in the laboratory, followed by field demonstrations at two sites.

Treatability studies were performed at the Louisiana State University to determine the optimum S/S agents and relative quantities to reduce the leaching potential of arsenic from the soil. Based on several successful case histories, portland cement was selected as the binder. Chemical additives tested in various ratios include Na_2SiO_3, K_2SiO_3, H_2O_2, $FeSO_4$, $FeCl_2$, $Fe_2(SO_4)_3$, $FeCl_3$, $MnCl_2$, $BaCl_2$, $MgCl_2$, $MgSO_4$, $MgCl_2/MgO$, $MnCl_2/MnO_2$, $BaCl_2/MgO$, CaO, attapulgite, bentonite, rice husk ash, and a commercial zeolite mix (Miller, 1996).

The use of portland cement alone as a binding agent significantly reduces arsenic leachability. Both small-scale (10 g) and larger scale (3 kg) tests showed that portland cement with ferrous sulfate ($FeSO_4$) lowers TCLP arsenic leachability more than the other binder/additive mixtures investigated. In addition, $FeSO_4$ treatment of the soil for five to seven days prior to the addition of portland cement consistently improved performance of the S/S soil treatment in these experiments (Miller, 1996).

Two sites were selected for field application of the S/S technology. The first site was underlain by silty and/or clayey sands (5 to 15% fines passing the No. 200 sieve), and the second was underlain by slightly silty sands (1 to 5% passing the No. 200 sieve). Greater than 20% fines can inhibit the S/S process by binding the mixing equipment. The sites must also allow accessibility and working space for the S/S equipment. Two types of equipment are available for in situ S/S field work: auger and rotary (Miller, 1996). Rotary was used in this application (Fig. 1), though the SC has used the auger method successfully at other sites.

The field demonstrations began in the fall of 1995 and were completed in about six weeks; the two sites were treated in sequence. Both sites were relatively small, that is, about 405 and 1012 m^2, respectively. Water tables at these two sites were relatively high, from about 0.3 to 2.5 m below the surface. As indicated by the treatability studies, ferrous sulfate slurry was mixed with the soil first, followed by a cement slurry 24 h later. At the first site, in situ S/S treatment extended up to 2.8 m below the surface, which resulted in about 765 m^3 of soil treated. At the second site, in situ treatment extended to depths as great as 16 feet, which produced about 2523 m^3 of treated soil (Miller, 1996).

Post-treatment testing (Table 1) showed that the projects were very successful. S/S

Fig. 1. In situ solidification/stabilization field application.

TABLE 1

Post-treatment test results, S/S with ferrous sulfate and portland cement (after Miller, 1996)

Soil/site	Laboratory test	Untreated soil	Treated soil from treatability study	Post-treatment field samples		
				28-day cure	90-day cure	180-day cure
Silty and/or clayey sand	TCLP for arsenic (mg/l)	1.42–3.7	0.18–02.9	ND–0.11	ND–0.08	<0.05
	Hydraulic conductivity (cm/s)	1×10^{-3} (approx.)	$1.0–1.7 \times 10^{-7}$	$0.5–0.64 \times 10^{-7}$	$1.47–1.9 \times 10^{-7}$	
Slightly silty sand	TCLP (mg/l) for arsenic	0.15–3.5	0.22–0.38	<0.05–0.12	<0.05–0.17	<0.05
	Hydraulic conductivity (cm/s)	5×10^{-3}	$0.7–1.9 \times 10^{-7}$	$0.5–2.2 \times 10^{-7}$	$0.47–1.6 \times 10^{-7}$	

treatment produced almost a 10-fold reduction in arsenic leachability, and four orders of magnitude reduction in permeability. Based on the post-treatment results and subsequent groundwater monitoring, both sites received a 'no further action' from state regulators. Total project costs, in 1995 dollars, were about \$110/m^3 treated, which is competitive with other treatment technologies (Miller, 1996).

ADVANCED PUMP AND TREAT

Soil Flushing

Soil flushing is the repeated passing of a solution through an aquifer in place, to remove the contamination from the soil in an abbreviated time frame. The solution can contain a reagent selected to remove the contaminants of interest, or flushing may be performed with water only. In this work, treatability studies were performed in the laboratory, fol-

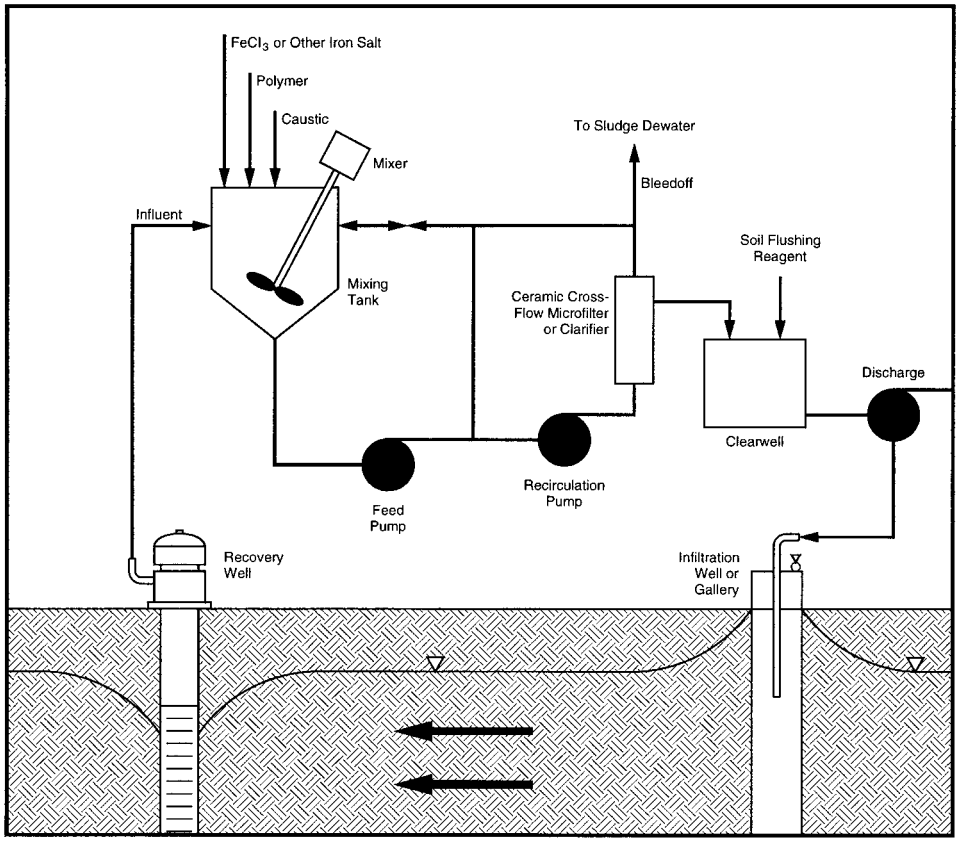

Fig. 2. Combined soil flushing/iron coprecipitation/ceramic membrane technology.

lowed by field demonstrations, which culminated in the first commercial application of soil flushing to clean a contaminated site.

Argonne National Laboratories performed initial treatability studies to determine the optimum flushing reagents for soils (Peters et al., 1994). Argonne investigated the following extractants: pH-adjusted water, sodium carbonate (Na_2CO_3), potassium phosphate dibasic (K_2HPO_4), citric acid, oxalic acid, phosphoric acid (H_3PO_4), polysodium vinyl sulfonate, and three commercially available extractants. Peters et al. (1994) describe the relative effectiveness of each of these reagents.

Citric acid was selected for field studies because it was both effective and environmentally friendly. In certain field applications, citric acid removed up to 100 times the amount of arsenic from soil than would be removed by water alone (Redwine and Peters, 1997). 0.01 M phosphoric acid was used in the commercial application described below, because that reagent was more compatible with the selected water treatment process.

Iron Coprecipitation and Cross-Flow Ceramic Membrane Filtration

In the iron coprecipitation process, an iron salt such as ferric chloride or ferric sulfate is added to contaminated water, and the pH adjusted (raised) to induce precipitation of iron oxyhydroxides (Fig. 2). During floc formation, trace elements such as arsenic adsorb

onto the iron floc. Iron coprecipitation has been proven effective for arsenic, cadmium, chromium, copper, lead, nickel, selenium, vanadium, and zinc.

Cross-flow ceramic membrane filtration can be used to separate the solids (floc) from the clean water in the iron coprecipitation treatment process (Fig. 2). The feed stream containing the floc enters multichanneled elements parallel to the membrane surface. Flow permeates into the filter perpendicular to the large channels, where it emerges as clean water. The ceramic membrane filter replaces a traditional clarifier in the system, and occupies much less space.

Case Histories

The SC combined soil flushing, iron coprecipitation, and ceramic membrane filtration (Fig. 2) to remediate an arsenic-contaminated site in Florida (Redwine and Peters, 1997). The site was underlain by a fine sand aquifer to a depth of about 6 to 7.7 m below ground surface, below which occurred a thin (1.5 m or less), but effective clay confining layer. A pumping test was first performed on the surficial aquifer to determine the optimum pumping rate, in this case about 45 l/min. A groundwater flow model was constructed for the aquifer, and pumping and injection scenarios tested in the model. Since soil flushing would be applied at this site, the pumping and injection system was designed to move the maximum amount of water through the aquifer in a given time frame, not to minimize the number of wells. An economic analysis showed that this approach would reduce total project costs.

From start-up throughout the life of the project, the water treatment system was effective in reducing arsenic concentrations to below the drinking water standard of 0.05 mg/l, and in many instances to below the detection limit of 0.005 mg/l (Fig. 3). Soil flushing at least doubled the amount of arsenic removed from the aquifer in a given time frame. After only six months of treatment, the contaminant plume had shrunk about 73% as compared to the historical maximum (Fig. 4). After 18 months of treatment, the plume stabilized at about 20% of its historical maximum. Environmental regulators granted a 'monitoring only' status for the site after four years of treatment, and the plume has not expanded since the treatment system was shut off (more than one year ago). The SC, in another iron coprecipitation project cofunded by EPRI, has implemented an improved, 284 to 322 l/min remediation system at another site. The improvements on the second generation system include larger size (scale-up), automation, computerization, horizontal wells to reach areas under energized equipment, data logging, off-site control, and several others.

ELECTROREMEDIATION

Electroremediation or electrokinetics, an emerging technology for environmental restoration, induces the movement of subsurface contamination under the influence of electrical fields, that is, a direct current between electrodes placed in the ground. Electroremediation variables include pH, voltage, amperage and chemical enhancements, as well as soil moisture and chemistry. The work described in this section represents a cooperative effort between the SC, Electrokinetics Inc. of Baton Rouge, Louisiana, and EPRI. Prior to this work, only one case history of electroremediation for arsenic existed, and it was not well-documented.

In a small study performed in 1997, the project team demonstrated that electroremediation could be used to effectively remove arsenic from contaminated soil. In this study, arsenic was extracted as an anion at the anode. Electroosmosis produced flow to the cathode, but arsenic did not accumulate at the cathode. The acidity generated by the electrokinetic process at the anode did not desorb the arsenic from the soils. An extractant, therefore, was used to release the arsenic from the soils (Redwine and Clark, 2000).

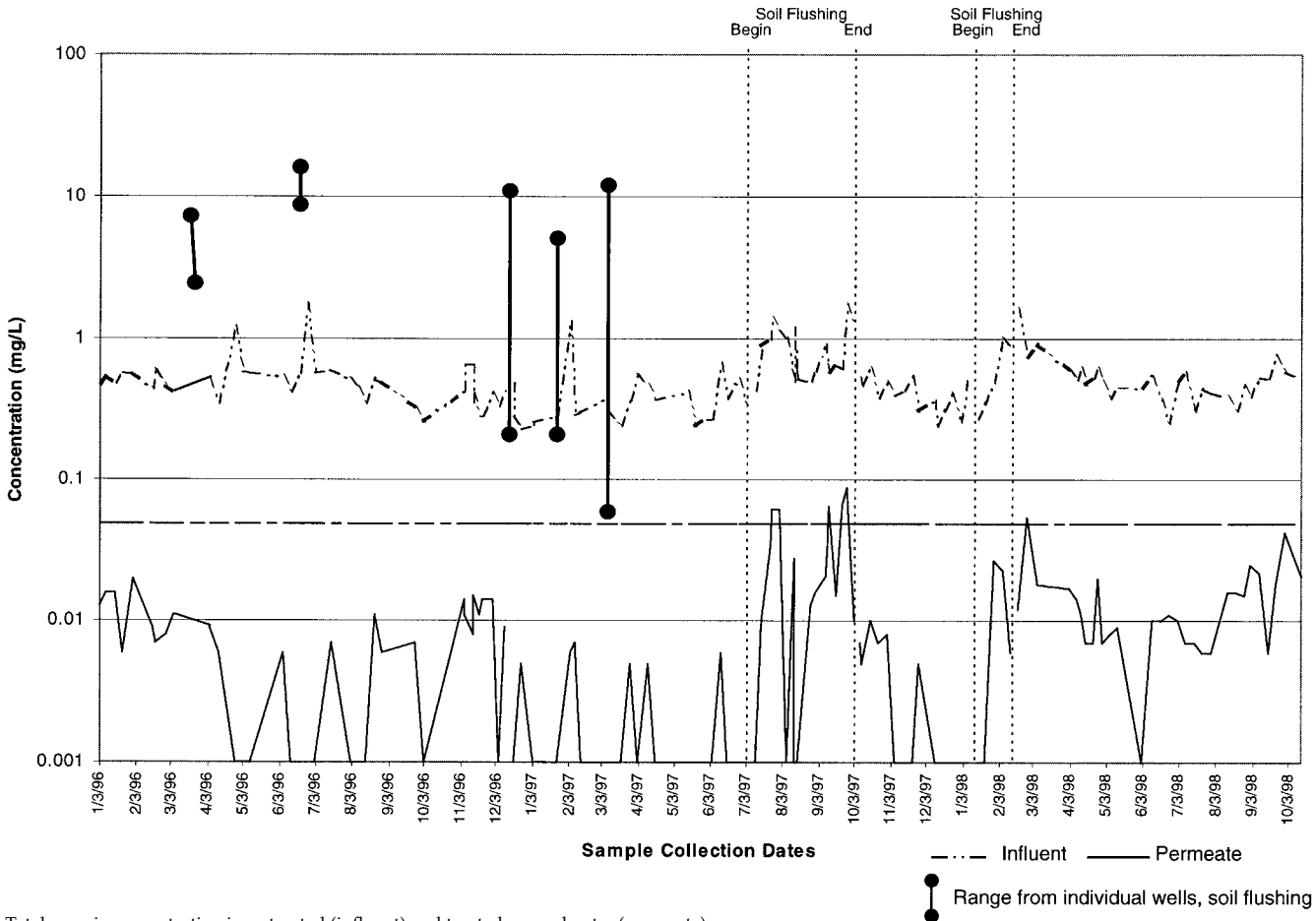

Fig. 3. Total arsenic concentration in untreated (influent) and treated groundwater (permeate).

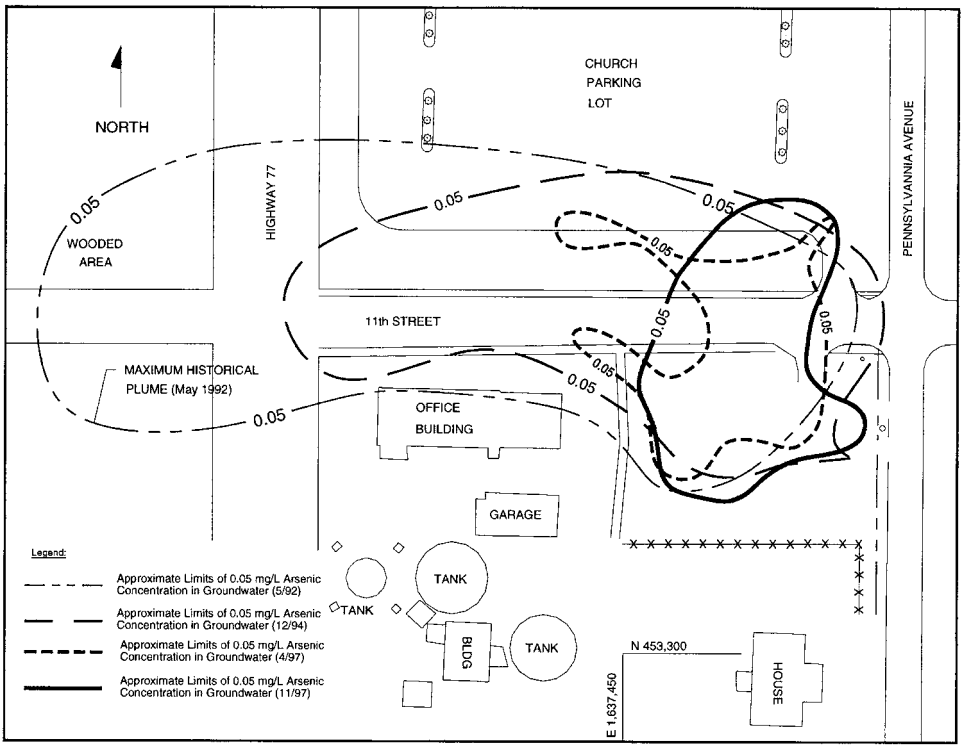

Fig. 4. Arsenic plume reduction through time, advanced pump and treat.

TABLE 2

Results of electroremediation treatability studies (after Electrokinetics, 1998)

	% to anode in 30 days	% to cathode in 30 days	Power requirements (kW h/m^3)
Unenhanced extraction	65	1	2969
Acid-amended extraction	27–90	0	101–798
Anode fence with sacrificial iron anode	77 electroplated, 23 in soil near anode	0	517
pH-controlled anode fence with sacrificial iron anode	25 electroplated, 75 in soil	0	120
Combined acid-amended and sacrificial iron anode	3 in iron–carbon bed, 97 stabilized in soil near anode. Leachability (TCLP, SPLP) reduced 4 to 5 times	0	1140

In subsequent studies, Electrokinetics Inc. performed five (5) bench-scale tests on arsenic-contaminated soils from substation sites: unenhanced extraction, acid-amended extraction, anode fence with sacrificial iron anode, pH-controlled fence with sacrificial iron anode, and combined acid-amended extraction and sacrificial iron anode (Electrokinetics, 1998). In the first two applications, arsenic would be extracted at the electrodes; in the other three applications, arsenic would be immobilized in the soil between the electrodes. Table 2 summarizes the results of these tests.

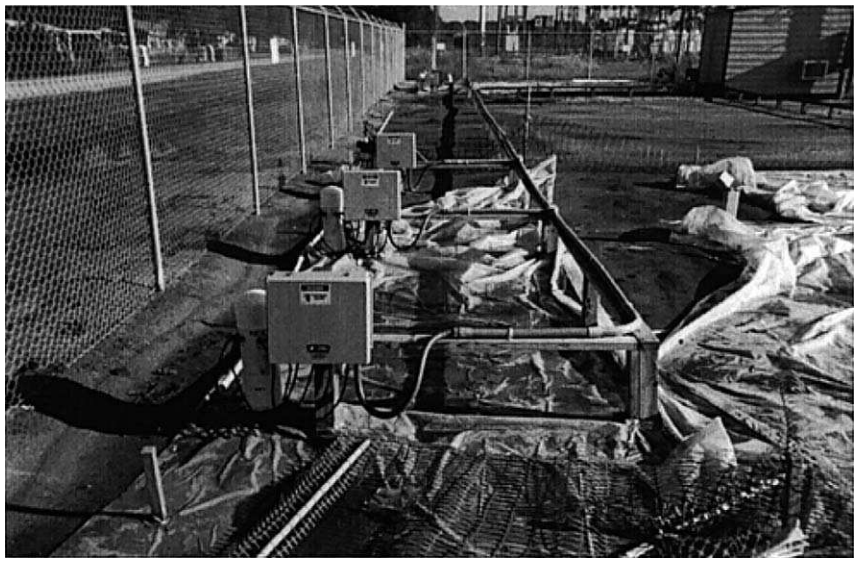

Fig. 5. Electroremediation field demonstration.

Based on the treatability study results, two test cells were constructed in the field at a contaminated site: an acid-amended extraction cell, and a combined acid-amended extraction/sacrificial iron anode cell (Fig. 5). Though remedial effectiveness monitoring data after one year are promising, they are not yet definitive. Evaluation of the electroremediation system is still under way, including a cost-comparison to other competing technologies. Based on laboratory results, soil treatment costs are expected to be less than $\$130/m^3$.

PHYTOREMEDIATION

Phytoremediation is the use of plants to clean contaminated soil and water. Some plants actually take up contaminants (e.g., metals and metalloids), and sequester them in their roots, leaves, or other tissues. These plants may then be harvested and disposed of. Plants that take up extraordinary amounts of contaminants are known as hyperaccumulators.

A literature search on plants and arsenic revealed that a number of plants, both aquatic and terrestrial, are known to accumulate or tolerate arsenic. In the southeastern U.S., the native plant moss verbena (*Verbena tenuisecta*) was observed growing on arsenic-contaminated sites. Though not observed growing directly on contaminated soils, the saw palmetto (*Sonoma repens*) thrives in the coastal southeastern U.S. The main objectives of this study were to determine if these two native plants could grow in high arsenic soils, and take up arsenic from the soil and bind it in their tissues. Since phosphate is known to release arsenic from soils, a second phase of the study examined the effect of high-phosphate fertilizer on the uptake of arsenic (Tinsley, 1999).

The first phase of the project confirmed that *V. tenuisecta* can grow in soil with up to 650 mg/kg arsenic after transplanting. (Seeds planted directly into contaminated soil germinated but died within a few days.) The transplants grew to maturity without showing stress, and continued to grow after harvest of the above-ground portions. *V. tenuisecta* took up arsenic into both the leaves and the roots. Based on a leaf analysis, the plants took up arsenic at a rate 20 to 75 times that of the control. Arsenic concentrations in the roots were even higher than in the leaves (Tinsley, 1999).

In the second phase of the project, application of a high-phosphate fertilizer increased the uptake of arsenic into *V. tenuisecta*. Arsenic concentrations in the whole plant were about 65 to 2900 times greater than the control. The *S. repens* accumulated arsenic only in the roots, at a rate of about 60 to 320 times greater than the control (Tinsley, 1999).

These experiments show that both these species could be used to stabilize arsenic-contaminated soil, thereby reducing contaminant transport by wind, surface runoff, or leaching to groundwater. *V. tenuisecta* will also take up some arsenic in the leaves, which could be periodically harvested to prevent return of arsenic to the soil. Both *V. tenuisecta* and *S. repens* are attractive species. *S. repens*, which is very tough and armed with spines, could be used to discourage foot traffic across contaminated sites (thereby reducing exposure). Since this plant takes up arsenic into the root but not the above-ground portions, no harvesting would be required (Tinsley, 1999).

IN SITU CHEMICAL FIXATION

In situ chemical fixation (ISF) is presently being investigated as a potential remediation technology for both soil and groundwater contaminated with arsenic. In this process, a liquid reagent would be applied to contaminated soil to immobilize the arsenic. Another reagent may be required to change the oxidation-reduction state of a cation to induce coprecipitation of the arsenic; this is known as redox manipulation (J.R. Howell in Hartsfield, 1999).

Variations of ISF processes have been applied to heavy metals in cationic form (e.g., Cd^{2+}, Cr^{3+}, Pb^{2+}), which reduced the toxicity and/or water solubility of the contaminant. Arsenic forms a number of relatively insoluble compounds with solubility products on the order of 10^{-10} to 10^{-51}. Research shows that arsenic compounds formed with ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron and calcium (Ca^{2+}) salts tend to be highly insoluble and relatively stable (J.R. Howell in Hartsfield, 1999).

Soil samples were collected in October 1999 for use in ISF treatability studies presently under way at the University of Alabama. The treatability studies involve both batch and column tests using soil and groundwater to determine the effectiveness of differing chemical reagents and their concentrations on the stabilization of arsenic. If the batch and column testing are successful, then a pilot scale application of the ISF technology may be conducted. The technology has potential for source fixation in the soil and prevention of groundwater contamination (J.R. Howell in Hartsfield, 1999).

NATURAL ATTENUATION MODELING

SC personnel observed significant reductions in arsenic in groundwater monitoring wells after source removal (or abatement) at several sites. In 1999, SC personnel searched for a groundwater model which would accurately predict the arsenic concentrations observed, for use in natural attenuation modeling and associated remedial action plans. After working with several candidate models, EPRI's MYGRT Version 3.0 was selected.

EPRI developed MYGRT to predict the migration of single component inorganic and organic solutes in unsaturated and saturated zones down gradient of sources (EPRI, 1998). The model allows for the evaluation of a number of remedial strategies. MYGRT is capable of predicting unsaturated zone transport, groundwater flow, and contaminant transport (EPRI, 1998; J.R. Howell in Lasher, 1999).

To date, MYGRT has been used to model three (3) sites. The model predicted actual plume configuration at all three sites using actual data or reasonable input parameters when data were not available. Source removal has been performed at one of the sites, and MYGRT accurately predicted the reductions in arsenic in groundwater monitoring wells after source removal.

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