4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

Extensive reviews of the metabolism of arsenic have been published recently (National Research Council, 1999, 2001; WHO, 2001). This section focuses on data relevant for the evaluation of carcinogenic effects. Thus, it is not a complete review on all published data. The oxidation state of arsenic and its metabolites are given if reported. If speciation of oxidation state has not been performed, the metabolites are given as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA).

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Absorption*

Arsenic in drinking-water is easily absorbed in the gastrointestinal tract. About 70–90% of a single dose of dissolved arsenite (As^{III}) or arsenate (As^{V}) was absorbed from the gastrointestinal tract of humans and experimental animals (Pomroy *et al*., 1980; Vahter & Norin, 1980; Freeman *et al*., 1995). A high rate of gastrointestinal absorption is also supported by the fact that people whose main fluid intake consists of drinking-water with elevated arsenic concentrations have very high concentrations of arsenic in their urine.

With regard to absorption of arsenic through the skin, a few experimental studies indicate a low degree of systemic absorption. Application of water solutions of radiolabelled arsenate *in vivo* to the skin of rhesus monkey and *in vitro* to human cadaver skin showed that about 2–6% and 0.98% of the applied arsenic was absorbed within 24 h, respectively (Wester *et al*., 1993). Similar in-vitro studies using dorsal skin of mice showed a much higher absorption: 33–62% of the applied dose of radiolabelled arsenate in aqueous solution was absorbed through the skin within 24 h. However, most of it, on average 60–90%, was retained in the skin (Rahman *et al*., 1994). These authors suggested that absorption of arsenic through the skin may be species-specific. For many chemicals, mouse skin is more permeable *in vitro* than human cadaver skin (Bronaugh *et al.*, 1982). In-vitro studies with human keratinocytes showed that 1–8% of the applied arsenic dose was retained per hour (Bernstam *et al*., 2002). Morphological changes, cytotoxicity and inhibition of DNA and protein syntheses were found with in-vitro doses of As^{III} as low as 10 µg/L. Thus, it seems probable that inorganic arsenic can be absorbed from the exterior, leading to a breakdown in skin barrier function.

In-vitro studies of skin absorption of DMA^{V} (10 µg in 20–100 µL water) by application to dorsal skin from adult mice mounted in flow-through diffusion cells showed that 16–25% was retained in the receptor fluid (Hanks balanced salt solution), about 15% in the skin and the remainder in the wash after 24 h (Hughes *et al*., 1995). After exposure

for 1 h only, essentially all of the applied dose was washed away. Less than 1% of the applied dose was absorbed.

A low degree of systemic skin absorption of inorganic arsenic is supported by studies showing that people in Fairbanks, AK, who used tap-water containing about 345 μg/L arsenic for washing, but only bottled water (which did not contain arsenic) for drinking, had about the same low concentrations of arsenic metabolites in urine (on average about 40 μg/L) as people with less than 50 μg/L in their tap-water (Harrington *et al*., 1978). The concentration of arsenic in hair was clearly elevated in the group drinking bottled water (5.74 μg/g compared with 0.43 μg/g in the low-arsenic group), which shows that arsenic is bound to hair and probably also to skin during washing with water rich in arsenic.

4.1.2 *Distribution*

Following its absorption, arsenate is rapidly reduced to As^{III}; the distribution of its metabolites in the body are therefore very similar to that following exposure to As^{III}. However, studies in mice given arsenite or arsenate (0.4 mg/kg bw) intravenously showed that the concentrations in stomach and intestines were higher after exposure to As^{III} than after exposure to As^{V}, while incorporation in bone was higher following exposure to As^{V} (Lindgren *et al*., 1982). The differences are less marked in the case of oral exposure, probably due to faster methylation that occurs when the absorbed arsenic passes directly to the liver. After exposure to toxic doses at which methylation capacity is exceeded or inhibited, the differences in distribution patterns for the two forms are greater (Vahter & Norin, 1980).

Absorbed arsenic is transported, mainly bound to SH groups in proteins and low-molecular-weight compounds such as glutathione (GSH) and cysteine, to different organs in the body (National Research Council, 1999, 2001). Complexation of trivalent arsenical compounds with GSH, probably mainly in the form of $As(GS)_3$, has been demonstrated, but As^{III} is easily transferred to binding sites of higher affinity, especially vicinal dithiols, such as lipoic acid and dimercaptosuccinic acid (Cullen & Reimer, 1989; Delnomdedieu *et al.*, 1993). Studies on serum arsenic in dialysis patients showed the presence of inorganic arsenic, partly bound to proteins, and DMA (Zhang *et al*., 1997, 1998a,b; De Kimpe *et al*., 1999). Transferrin was the main carrier protein, but the extent to which this occurs in healthy individuals is not known. Most of the arsenic in blood is rapidly cleared, following a three-exponential clearance curve (Mealey *et al*., 1959; Pomroy *et al*., 1980). The majority of arsenic in blood is cleared with a half-time of about 2 or 3 h. The half-times of the second and third phases are about 168 and 240 h, respectively (Mealey *et al.*, 1959; National Research Council, 1999).

In experimental studies on mammals exposed to inorganic arsenic, the tissues with the longest retention of arsenic, depending on species, were skin, hair, liver, kidney, blood, squamous epithelium of the upper gastrointestinal tract, epididymis, thyroid, skeleton and lens. Arsenic does not readily cross the blood–brain barrier, and concentrations in the brain are generally low compared with most other tissues (Lindgren *et al*., 1982; Vahter *et al*., 1982; Lindgren *et al*., 1984; Yamauchi & Yamamura, 1985).

In human subjects exposed chronically to arsenic and also at background environmental concentrations, the hair and nails generally show the highest concentrations (0.02–10 mg/kg dry wt; Hindmarsh, 2002). Thus, arsenic appears to concentrate in tissues with a high content of cysteine-containing proteins. In areas of West Bengal and Bangladesh that have high concentrations of arsenic in the drinking-water, maximal concentrations of arsenic in hair, nail and skin exceeding 40 mg/kg have been reported (Guha Mazumder *et al.*, 1988; Chowdhury *et al*., 2001; Basu *et al.*, 2002). Very few studies have been carried out on the distribution of arsenic in human tissues. Postmortem analysis of human tissues confirm that arsenic is widely distributed in the body after long-term exposure, with highest concentrations in the skin and lungs (0.01–1 mg/kg dry wt), as well as hair and nails (Liebscher & Smith, 1968; Cross *et al.*, 1979; Dang *et al*., 1983). In people exposed to high concentrations (0.2–2 mg/L) of arsenic in drinking-water, the concentration in liver was 0.6–6 mg/kg dry wt compared with 0.16 mg/kg in unexposed people (Guha Mazumder *et al*., 1988). In a case of acute intoxication by arsenic, the liver and kidneys showed the highest concentrations of total arsenic with values 350- and 63-fold higher than those in blood, respectively. In all organs, As^{III} was the predominant species, and MMA occurred at higher concentrations than DMA. MMA and DMA were more prevalent in lipid-rich organs (49% and 45% of total arsenic in cerebellum and in brain, respectively) compared with other organs (~ 20% of total arsenic). As^{V} was found in small quantities in the liver, kidneys and blood (2% of total arsenic) (Benramdane *et al*., 1999).

Dang *et al*. (1983) used neutron activation analysis (NAA) to measure total arsenic in tissues of people [age and sex not specified] dying in accidents in Mumbai, India (Table 29). Concentrations in the brain were generally low compared with most other tissues. Thus, it appears that arsenic does not readily cross the blood–brain barrier. Notably, there was a large variation in tissue concentrations of arsenic among individuals, similar to that reported in earlier studies (Liebscher & Smith, 1968; Larsen *et al.*, 1974).

Table 29. Levels of arsenic in human tissues obtained from traffic accident victims in the Mumbai area of India

Tissue	No. of samples	Mean concentration (± SD) of arsenic (mg/kg wet wt)
Brain	12	3.9 ± 1.0
Blood	8	5.9 ± 3.9
Kidney	13	12.4 ± 20.7
Liver	19	14.5 ± 6.9
Spleen	18	15.2 ± 16.6
Lung	13	19.9 ± 22.7

From Dang *et al*. (1983)

Few studies have examined the distribution of arsenic metabolites in tissues, owing to analytical difficulties. Marafante *et al*. (1982) reported predominantly inorganic arsenic in ultrafiltrates of rat and rabbit liver and kidney 1 h after intraperitoneal injection of 50 µg/kg bw [^{74}As] as sodium arsenite, using ion-exchange chromatography with radiometric detection. The fraction present as MMA was generally less than one-tenth that of inorganic arsenic. De Kimpe *et al*. (1996) studied the tissue distribution of arsenic metabolites up to 120 h after intraperitoneal injection of a trace amount of [^{74}As]-arsenate in male Flemish giant rabbits, also using ion-exchange chromatographic separation of ultrafiltrates with radiometric detection. The predominant metabolite present in tissues was DMA, followed by inorganic arsenic species and low concentrations of MMA. The percentage of DMA increased steadily over time in bone marrow, heart, liver, muscle, pancreas, small intestine and spleen, but levelled off or declined in kidney and lung.

Yamauchi and Yamamura (1985) studied the tissue distribution over time of arsenic metabolites in male Syrian golden hamsters given a single oral dose of 4.5 mg/kg bw arsenic trioxide. Speciation of arsenic metabolites was carried out by hybrid generation–atomic absorption spectrophotometry (HG–AAS) with a cold trap after alkaline digestion. The predominant form of arsenic present in all tissues up to 120 h after dosing was inorganic arsenic. In contrast to other studies, the concentrations of MMA in tissue were two- to fourfold higher than those of DMA at all time-points, while much more DMA (22% of the dose in 5 days) than MMA (2.5% of the dose) was excreted in urine. The highest concentrations of MMA were found in lungs and spleen at 12–24 h, and those of DMA in liver, lung and kidney at 24 h.

Yamauchi *et al*. (1988) reported data on the time-course tissue distribution in hamsters given a single oral dose of 50 mg/kg bw MMA. Peak MMA concentrations were achieved within 6–120 h after dosing and were highest in the kidney, followed by spleen, lung, skin, liver, muscle and brain. MMA itself accumulated in the kidney and levels declined very slowly. DMA was also detected in several tissues, with highest levels occurring in the lung, followed by kidney and liver. Trimethylated arsenic was not detected in any tissues.

The fate of DMA has been studied in mice administered [^{74}As] or [^{14}C]DMA intravenously. The highest levels of radioactivity were present in kidney at all time-points (5–60 min after injection). Tissues that retained arsenic for the longest time (24 h) were the lungs, intestinal walls, thyroid and lens (Vahter *et al*., 1984; Hughes *et al*., 2000). Yamauchi and Yamamura (1984) studied the tissue distribution of DMA in hamsters administered a single oral dose of 50 mg/kg bw DMA. Concentrations were elevated in all tissues examined, including the brain, indicating that DMA is widely distributed in the body and that it passes the blood–brain barrier, although not to a large extent. Concentrations of DMA peaked at 6 h in all tissues examined except hair, with the highest levels in lung, followed by kidney, spleen, liver, skin, muscle and brain. Part of the DMA was found to be methylated further to trimethylarsenic (TMA) *in vivo*. Concentrations of TMA peaked at 6 h in all tissues except skin and hair in which none was detected. The highest concentrations of TMA were found in lung, and were equivalent to about half of those of DMA. It is notable that the peak concentrations of DMA and TMA in the lung were over

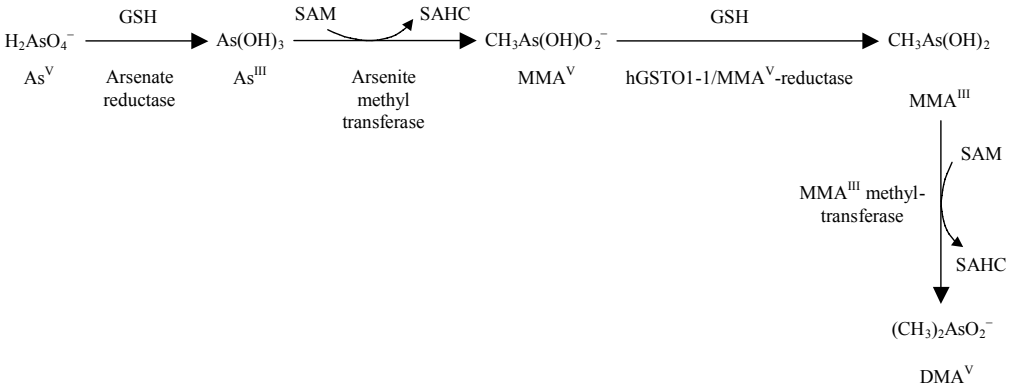
fourfold higher than those in the liver and kidney, but at 120 h after dosing, both had declined to control levels. The authors presumed that the TMA biosynthesized by hamsters is more likely to behave as an organic arsenic compound, such as arsenobetaine which is present in seafoods, than to be a very toxic substance such as trimethylarsine.

4.1.3 *Metabolism*

(*a*) *Methylation of arsenic*

For several decades, it has been known that inorganic arsenic is metabolized via methylation in microorganisms, aquatic organisms, birds and mammals. The methylation occurs through alternating reductive and oxidative methylation reactions, that is, reduction of pentavalent to trivalent arsenic followed by addition of a methyl group (Figure 3). In certain microorganisms, the methylation of inorganic arsenic may proceed to trimethylated metabolites. In humans, the relative amounts of species in urine are generally 10–30% inorganic arsenic, 10–20% MMA_{total} and 60–80% DMA_{total} (Hopenhayn-Rich *et al.*, 1993; National Research Council, 1999; Vahter, 1999a) (see below for further discussions of variations). The main metabolites excreted in the urine of humans exposed to inorganic arsenic are mono- and dimethylated arsenic acids, together with some unmetabolized inorganic arsenic. The major urinary methylated metabolites of arsenic are MMA^V and DMA^V, with arsenic in its pentavalent oxidation state. However, recent studies have demonstrated MMA^V reductase activity in different tissues that gives rise to the presence of both monomethylarsonous acid (MMA^{III}) and/or dimethylarsinous acid (DMA^{III}) in hamster liver (Sampayo-Reyes *et al.*, 2000) and of MMA^{III} in the bile of various experimental

Figure 3. Biotransformation of inorganic arsenic



Adapted from Zakharyan *et al.* (2001)

The conjugate acids and bases of the several forms of arsenic that are thought to predominate at physiological pH are shown.

SAM, *S*-adenosyl-L-methionine; SAHC, *S*-adenosyl-L-homocysteine; hGSTO1-1, human glutathione-*S*-transferase omega 1-1 (which is identical to MMA^V-reductase)

animal species (Csanaky & Gregus, 2002), as well as DMA^{III} and/or MMA^{III} in human urine (Aposhian *et al*., 2000a; Le *et al*., 2000b; Del Razo *et al*., 2001a; Mandal *et al*., 2001) after exposure to inorganic arsenic.

Following exposure of mice, hamsters, rats and humans to DMA^{V}, further methylation to trimethylarsine oxide (TMAO) has been observed (Marafante *et al*., 1987; Yoshida *et al*., 1997; Kenyon & Hughes, 2001). This probably occurs via DMA^{III} or the DMA-complex observed in urine. About 5% of urinary arsenic was in the form of TMAO. Of the species studied, demethylation of DMA to inorganic arsenic was detected only in rats, in which inorganic arsenic constituted less than 10% of the total excreted (Yoshida *et al*., 1997). Inorganic arsenic appeared in urine during the first 24–48 h after administration, whereas the highest rate of excretion of unchanged DMA had occurred by 6 h and that of TMAO between 6 and 24 h after dosing. The authors suggested that demethylation of DMA was effected by intestinal flora. TMAO was not found in an in-vitro study after incubation of inorganic arsenic with rat liver cytosol. Indeed, it was shown that the methylation of MMA to DMA was inhibited by increasing concentrations of As^{III} preventing the formation of TMAO (Buchet & Lauwerys, 1985, 1988).

It should be noted that there are pronounced species differences in the metabolism of arsenic (National Research Council, 1999; Vahter, 1999b). Most experimental animals excrete very little MMA in urine compared with humans (Vahter, 1999b), and some animals, in particular guinea-pigs and several species of non-human primates (Vahter, 1999b; Wildfang *et al*., 2001), are unable to methylate inorganic arsenic at all. In addition, rats show different kinetics of arsenic metabolism with a pronounced accumulation of DMA^{III} in red blood cells (Shiobara *et al*., 2001) and greater biliary excretion of arsenic (Klaassen, 1974; Gregus *et al*., 2000), compared with humans. The unique disposition of arsenic in rats may be due to the pronounced biliary excretion of MMA^{III} and blood cell uptake of DMA^{III} (Gregus *et al*., 2000; Shiobara *et al.*, 2001). Thus, it is difficult to evaluate human metabolism of arsenic based on much of the available experimental animal data. Studies in hamsters and rabbits seem to be the most useful because their metabolism is most similar to that in humans (National Research Council, 1999). This phenomenon has been taken into consideration here, and data from rats are not included, except for some information from in-vitro studies with rat hepatocytes, which has been used for the purpose of adding mechanistic information, where appropriate.

Compared with inorganic arsenic, the methylated metabolites containing pentavalent arsenic (MMA^{V} and DMA^{V}) are less cytotoxic, less reactive with tissue constituents and more readily excreted in urine (for review see National Research Council, 1999, 2001; Vahter & Concha, 2001). This has been taken as evidence that methylation of arsenic is an efficient detoxification process. In general, trivalent arsenic is more toxic than the pentavalent form. Recent studies, however, show that the trivalent methylated metabolites are considerably more toxic than inorganic As^{III} (National Research Council, 1999, 2001; Thomas *et al*., 2001; Sections 4.2 and 4.4). Thus, their presence in tissues and body fluids implies that the metabolism of inorganic arsenic involves important bioactivation processes, and that the toxicity of inorganic arsenic probably depends on its metabolism,

especially the capacity of cells to produce methylated intermediates that react with tissue constituents. It should be noted that there may be other mechanisms of transport out of tissues to urine. Excretion of arsenic in chimpanzees was found to be more rapid than that in humans, although methylation of arsenic does not occur in chimpanzees (Vahter *et al*., 1995a). A more complete understanding of the mechanisms of the metabolism of arsenic will provide further insight into the factors determining susceptibility to its toxicity. It should be noted that it is difficult to evaluate the tissue concentrations of MMA^{III} and DMA^{III} based on the amounts detected in urine.

A few studies have indicated a slightly larger fraction of urinary MMA and a smaller fraction of DMA in people with arsenic-related health effects, including skin lesions (Del Razo *et al*., 1997; Yu *et al*., 2000) and chromosomal aberrations (Mäki-Paakkanen *et al*., 1998). Similarly, there are indications that a relatively large amount of MMA in urine is associated with greater retention of arsenic in the body. Evaluation of data from a number of experimental studies on humans receiving specified doses of inorganic arsenic indicates that a higher percentage of DMA in urine is associated with greater overall excretion, while a higher percentage of inorganic arsenic and MMA is associated with slower excretion of total arsenic metabolites (Vahter, 2002). It should also be noted that other mammals that excrete little (rat, rabbit, hamster, beagle and mouse) or no MMA (marmoset, chimpanzee and guinea-pig) in the urine, that is, most experimental animals, show a rapid overall excretion of arsenic (Vahter, 1999b). They also seem to be less susceptible than humans to arsenic-induced toxicity, including cancer (National Research Council, 1999).

(*b*) *Mechanism of methylation of arsenic*

The mechanism of methylation of arsenic in humans has not been elucidated, but *S*-adenosylmethionine (SAM) seems to be the main methyl donor. In experimental studies, inhibition of SAM-dependent methylation pathways (by periodate-oxidized adenosine [PAD] or *S*-adenosylhomocysteine [SAH]) resulted in a marked decrease in methylation of arsenic (Marafante *et al*., 1985; De Kimpe *et al*., 1999; Csanaky & Gregus, 2001). Rabbits fed diets with a low content of methyl groups (low in methionine, protein or choline) methylated arsenic to a lesser degree (Vahter & Marafante, 1987). In-vitro studies using rat liver preparations have confirmed the requirement of SAM and thiols (reduced GSH) in the formation of MMA and DMA from As^{III} (Buchet & Lauwerys, 1988; Styblo *et al*., 1996; Healy *et al*., 1999; Thomas *et al*., 2001).

As shown in Figure 3, the methyl groups are transferred from SAM to arsenic in its trivalent form. GSH or other thiols serve as reducing agents for As^{V} and MMA^{V} (National Research Council, 1999, 2001) and are required for the methylation of arsenic. Complexation of trivalent arsenic with GSH, probably mainly in the form of $As(GS)_3$, has been demonstrated. Depletion of hepatic GSH by buthionine sulfoximine in rats and hamsters has been shown to decrease the methylation of inorganic arsenic (Buchet & Lauwerys, 1988; Hirata *et al*., 1990). Although As^{V} may be reduced non-enzymatically by GSH, enzyme-catalysed reduction seems to predominate. Studies with mice, rabbits,

and marmoset monkeys showed that a substantial fraction of absorbed arsenate (As^{V}) is rapidly reduced, probably mainly in the blood, to As^{III} (Marafante *et al.*, 1985; Vahter & Marafante, 1989; Vahter, 2002). Arsenate and pentavalent methylated metabolites may also be reduced to the corresponding trivalent form in tissues. Arsenate reductase activity has been detected in human liver (Radabaugh & Aposhian, 2000) and MMA^{V} reductase in human activity and rabbit liver (Zakharyan & Aposhian, 1999; Zakharyan *et al*., 2001) and various hamster tissues (Sampayo-Reyes *et al*., 2000). There is evidence that human MMA^{V} reductase is identical to glutathione-*S*-transferase class omega 1-1 (GSTO1-1) (Zakharyan *et al*., 2001). Based on studies on rabbit liver, it appears that reduction of MMA^{V} is the rate-limiting step in the metabolism of arsenic (Zakharyan & Aposhian, 1999). In male hamsters, MMA^{V} reductase activity was found to vary considerably among tissues: the highest activity was found in the brain followed by urinary bladder, spleen, liver, lung, heart, skin, kidney and testis. The activity in the testis was only about 10% of that in the brain (Sampayo-Reyes *et al*., 2000).

Experimental studies conducted in rabbits have indicated that the liver is the main site of arsenic methylation, especially following ingestion, when the absorbed arsenic initially passes through the liver, the only organ in which DMA is present 1 h after administration of the parent compound (Marafante *et al*., 1985). This is supported by studies showing a marked improvement in the methylation of arsenic following liver transplantation in patients with end-stage liver disease (Geubel *et al*., 1988). In-vitro studies have shown that the methylating capacity of different tissues may vary considerably. Investigation of the methylating activity of arsenic in male mice showed that the highest activity occurred in the testes, followed by kidney, liver and lung (Healy *et al*., 1998). The situation in female animals or humans remains to be elucidated. DMA was the main excretory metabolite of rat and human hepatocytes exposed to inorganic arsenic *in vitro* (Styblo *et al*., 1999). In addition to arsenic methyltransferase activity, the tissue in which arsenic is methylated may also depend on the cellular uptake of its different forms. Experimental studies show a several-fold higher uptake of As^{III} and MMA^{III} than of As^{V} and MMA^{V} in liver cells (National Research Council, 1999; Styblo *et al.*, 1999, 2000; National Research Council, 2001). In contrast, arsenate is readily taken up in the kidneys, after which it can be reduced and excreted in the urine, partly in methylated form. Whether this also occurs with MMA^{V} and DMA^{V} is not known. The uptake of MMA^{V} and DMA^{V} in most other tissues of mice seems to be low (Hughes & Kenyon, 1998; National Research Council, 2001).

The methyltransferases involved in arsenic methylation have not been fully characterized, although enzymes from liver cells of rats, rabbits, hamsters and rhesus monkeys have been partially characterized (Zakharyan *et al*., 1995, 1996; Wildfang *et al*., 1998; Lin *et al.*, 2002) as cytosolic enzymes of 46–60 kDa, the activity of which requires both SAM and a thiol. As^{III} and MMA^{V} methylating activities seem to involve the same protein. Arsenate, selenate, selenite and selenide were not methylated by the purified enzyme preparations (Zakharyan *et al.*, 1995). Arsenite and $MAs^{III}O$ (methylarsine oxide) methyltransferase activities have been detected in primary cultures of human hepatocytes (Styblo *et al*., 1999). The mRNA for As^{III} methyltransferase, purified from liver cytosol of male

rats, was found to be similar to Cyt19, a putative methyltransferase expressed in human and mouse tissues (Lin *et al*., 2002), and was detected in rat tissues and in HepG2 cells, a human cell line that was reported to methylate arsenic, but not in UROtsa cells, an immortalized human urothelial cell line that does not methylate arsenite.

(*c*) *Variation in arsenic metabolism*

Although a number of studies have shown that the average relative distribution of arsenic metabolites in the urine is 10–30% inorganic arsenic, 10–20% MMA_{total} and 60–70% DMA_{total} (calculated percentages) (National Research Council, 1999), there is a wide variation among individuals (Vahter, 1999a,b). In one study, interindividual variation was found to exceed intra-individual variation considerably; the efficiency of arsenic methylation of an individual is remarkably stable over time (Concha *et al.*, 2002). Also, a few recent studies in which the trivalent and pentavalent metabolite forms have been speciated indicate a considerable variation in urinary excretion of MMA^{III} and DMA^{III} (Aposhian *et al.*, 2000a; Del Razo *et al*., 2001b; Mandal *et al*., 2001). Differences between population groups have also been reported. In one study, indigenous people living in the Andes, mainly Atacameños, excreted less MMA in urine (often only a few per cent) (Vahter *et al*., 1995b); people living in certain areas of Taiwan, China, however, seem to have an unusually high percentage of MMA in urine (20–30% on average) (Chiou *et al*., 1997b; Hsueh *et al.*, 1998). These findings indicate that the influence of genetic polymorphims is more important than environmental factors for the variation in arsenic methylation. Recently, it was reported that the methylation pattern among 11 families in Chile correlated more strongly between siblings than between father–mother pairs (Chung *et al*., 2002), supporting a genetic basis for the variation in arsenic methylation.

A few human studies indicate that high doses of arsenic may influence its methylation in humans. In humans acutely intoxicated by inorganic arsenic, there is a marked delay in the urinary excretion of DMA which exceeds all other metabolites (Mahieu *et al*., 1981; Foà *et al*., 1984). Only after 1 or 2 weeks did the fraction of DMA in urine reach 70–80%, a level commonly seen after lower exposures. In people exposed to arsenic via drinking-water, the ratio of DMA to MMA in urine decreased somewhat with increasing level of exposure (Hopenhayn-Rich *et al*., 1993). This is probably related to inhibition of methyltransferase, especially in the second methylation step, by high concentrations of arsenite, as demonstrated in experimental studies *in vitro* (Buchet & Lauwerys, 1988; Styblo *et al*., 1996). In people exposed to high concentrations of arsenic in drinking-water (several hundred micrograms per litre), there is a slight decrease in the percentage of DMA and a corresponding decrease in the percentage of MMA (Hepenhayn-Rich *et al*., 1993). In some studies, women tend to methylate arsenic more efficiently than men (Hopenhayn-Rich *et al*., 1996c), which may in part be related to the observed increase in arsenic methylation during pregnancy (Concha *et al*., 1998c).

4.1.4 *Placental transfer*

Studies in experimental animals and humans show that both inorganic arsenic and methylated metabolites cross the placenta to the fetus (Concha *et al*., 1998c). In women exposed to arsenic in drinking-water (about 200 µg/L), the concentrations of arsenic in umbilical cord blood were about as high as those in maternal blood in late gestation (about 10 µg/L) (Concha *et al*, 1998c). Placentas also had elevated concentrations of arsenic (median, 34 µg/kg wet wt; range, 17–54 µg/kg; $n = 11$). More than 90% of the arsenic in urine and plasma of both newborns and their mothers (at the time of delivery) was in the form of DMA (compared with about 70% in non-pregnant women), indicating an increase in arsenic methylation during pregnancy. The authors suggested that the DMA is much less toxic to the embryo and fetus than inorganic arsenic; the increased arsenic methylation during pregnancy could be highly protective for the developing organism.

Studies on women living in north-western Argentina indicated a low degree of arsenic excretion in human breast milk (Concha *et al*., 1998a). The average concentration of arsenic in milk was 2 µg/kg fresh wt, compared with 10 µg/L in maternal blood and 320 µg/L in maternal urine. Breastfeeding of newborns from highly exposed areas decreased the levels of arsenic in their urine (which were elevated directly after birth) because of the low concentration in maternal breast milk (compared with formula milk prepared from the local water, which would provide about 200 µg arsenic/day) (Concha *et al*., 1998c). A study of 36 German women showed that the average concentration of total arsenic in breast milk was less than 0.3 µg/L (Sternowsky *et al*., 2002). The few women who reported a high intake of seafood showed increased arsenic levels in breast milk, indicating that organic arsenic compounds of marine origin, e.g. arsenobetaine, are excreted in milk.

4.1.5 *Excretion*

In humans, the major route of excretion of most arsenic compounds is via the urine. The biological half-time of inorganic arsenic is about 4 days, but is slightly shorter following exposure to arsenate than to arsenite (Crecilius, 1977; Yamauchi & Yamamura, 1979; Tam *et al*., 1979; Pomroy *et al*., 1980; Buchet *et al*., 1981). In six human subjects who ingested radiolabelled [^{74}As]arsenate, 38% of the dose was excreted in the urine within 48 h and 58% within 5 days (Tam *et al*., 1979). The results of another study indicate that the data were best fit to a three-compartment exponential model, with 66% excreted with a half-time of 2.1 days, 30.4% with a half-time of 9.5 days and 3.7% with a half-time of 38.4 days (Pomroy *et al*., 1980). In three subjects who ingested 500 µg arsenic in the form of arsenite in water, about 33% of the dose was excreted in the urine within 48 h and 45% within 4 days (Buchet *et al*., 1981).

The administration of sodium 2,3-dimercapto-1-propane sulfonate (DMPS), a chelating agent, to humans chronically exposed to inorganic arsenic in the drinking-water resulted in increased urinary excretion of arsenic (Aposhian *et al*., 2000b; Guha Mazumder *et al*., 2001a). In particular, there was a marked increase in urinary excretion of MMA^{III} and

MMA^{V}, while the concentration and percentage of urinary DMA decreased (Aposhian *et al*., 2000b). Experimental studies supported the hypothesis that DMPS competes with endogenous ligands for MMA^{III}, forming a DMPS–MMA complex that is not a substrate for the MMA^{III} methyltransferase enzyme. This may explain the decrease in the conversion of the MMA^{III} to DMA. The DMPS–MMA complex is readily excreted in urine. Interestingly, MMA^{III} was excreted in the urine only after administration of DMPS (Aposhian *et al.*, 2000b).

4.2 Toxic effects

4.2.1 *Humans*

(*a*) *Acute and subacute toxicity*

Acute effects caused by the ingestion of inorganic arsenic compounds, mainly As^{III} oxide, are well documented. The major lesion is profound gastrointestinal damage, resulting in severe vomiting and diarrhoea, often with blood-tinged stools — symptoms that resemble cholera. Other acute symptoms and signs include muscular cramps, facial oedema and cardiac abnormalities; shock can develop rapidly as a result of dehydration. Subacute effects mainly involve the respiratory, gastrointestinal, cardiovascular, nervous and haematopoietic systems (WHO, 1981).

(*b*) *Chronic toxicity*

Most of the reports on chronic exposure to arsenic in humans focus attention on skin manifestations because of their diagnostic specificity. However, data derived from population-based studies and clinical case series and reports relating to intake of inorganic arsenic through drinking-water, medications or occupational and environmental exposure show that chronic exposure to arsenic adversely affects multiorgan systems. The clinical appearance of non-cancerous manifestations of arsenic intoxication in humans is insidious in onset and is dependent on the magnitude of the dose and the time course of exposure.

(i) *Cutaneous manifestations*

The specific cutaneous lesions of chronic arsenic toxicity are characterized by pigmentation and keratosis. These have been reported from different regions of the world including Argentina, Bangladesh, Chile, China, India (West Bengal), Japan, Mexico and Taiwan, China, where the content of arsenic in drinking-water is elevated (Zaldívar, 1974; Borgoño *et al*., 1977; Cebrián *et al*., 1983; Saha, 1984; Chakraborty & Saha, 1987; Guha Mazumder *et al*., 1988, 1992; Ahmad *et al*., 1997; Guha Mazumder *et al*., 1998b; Biswas *et al*., 1998; Mandal *et al*., 1998; Ahmad, S.A. *et al*., 1999; Milton & Rahman, 1999; Guo *et al*., 2001). The magnitude of dose and the time frame of exposure to arsenic needed to induce the hyperpigmentation and hyperkeratosis characteristic of chronic arsenic intoxication have been investigated to a limited extent.

Among the population exposed to arsenic in drinking-water in the Antofagasta region of Chile, where levels reached 0.8 mg/L, cases of cutaneous arsenicosis, including both hyperpigmentation and hyperkeratosis, have been described in children as young as 2 years of age (Rosenberg, 1974). In a cohort of 40 421 inhabitants of south-western Taiwan, China, investigated by Tseng *et al*. (1968), the youngest subjects found to have hyperpigmentation and hyperkeratosis were reported to be aged 3 and 4 years, respectively; in a later investigation, the youngest subjects were aged 5 and 15 years (Tseng, 1977). The amount of arsenic consumed by these children was not specified. In a clinical evaluation conducted among 296 residents of Region Lagunera in northern Mexico, where ingested groundwater contained a mean arsenic concentration of approximately 0.4 mg/L, the shortest time of exposure associated with hypopigmentation was 8 years, increasing to 12 years for hyperpigmentation and palmoplantar keratosis (Cebrián *et al*., 1983).

The hyperpigmentation of chronic arsenic poisoning commonly appears in a finely freckled, raindrop pattern that is particularly pronounced on the trunk and extremities, and distributed bilaterally symmetrically, but can also involve mucous membranes such as the undersurface of the tongue or buccal mucosa (Yeh, 1973; Tay, 1974; Saha, 1984; Guha Mazumder, 1988, 1992; Saha, 1995; Guha Mazumder *et al.*, 1998b; Ahmad, S.A. *et al*., 1999; Milton & Rahman, 1999). Although less common, other patterns include diffuse hyperpigmentation (melanosis) (Tay, 1974; Saha, 1984), localized or patchy pigmentation, particularly affecting skinfolds (Tay, 1974; Szuler *et al*., 1979), and so-called leukodermia or leukomelanosis (Saha, 1984; Mandal *et al*., 1996, 1997; Chowdhury *et al*., 2000a,b) in which the hypopigmented macules take a spotty, white appearance.

Arsenical hyperkeratosis appears predominantly on the palms of the hands and on the plantar aspect of the feet, although involvement of the dorsum of the extremities and the trunk have also been described. Occasionally, lesions may be larger and have a nodular or horny appearance. In severe cases, the hands and soles present diffuse verrucous lesions. Cracks and fissures may be severe on the soles (Sommers & McManus, 1953; Black, 1967; Tseng *et al*., 1968; Yeh, 1973; Tay, 1974; Zaldívar, 1974; Borgoño *et al*., 1977; Cebrián *et al.*, 1983; Saha, 1984; Guha Mazumder *et al*., 1988; Ahmad *et al*., 1997; Guha Mazumder *et al*., 1998b; Saha & Chakraborti, 2001). Histological examination of the lesions typically reveals hyperkeratosis with or without parakeratosis, acanthosis and enlargement of the rete ridges. In some cases, there may be evidence of cellular atypia (mitotic figure) in large vacuolated epidermal cells (Tay, 1974). Yeh (1973) classified arsenical keratosis into two types: a benign type A (further subgrouped into those with no cellular atypia and those with mild cellular atypia); and a malignant type B (intraepidermal carcinoma or carcinoma *in situ*, basal-cell carcinoma or squamous-cell carcinoma). Type B arsenical keratosis is histologically similar to but not indistinguishable from Bowen disease. Skin cancer can arise in the hyperkeratotic areas or appear on non-keratotic areas of the trunk, extremities or hands (Sommers & McManus, 1953; Yeh, 1973). In epidemiological studies in West Bengal (India) and Bangladesh, a higher prevalence of arsenical skin lesions was observed in men compared with women, with a clear

dose–response relationship (Guha Mazumder *et al*., 1998c; Rahman *et al*., 1999a; Tondel *et al*., 1999).

Early studies provided estimates for the dose–response relationship of arsenic-induced skin lesions. Tseng *et al*. (1968) and Yeh (1973) evaluated 40 421 inhabitants of south-western Taiwan, China, where the drinking-water supply (artesian well-water) had been contaminated with arsenic for more than 50 years. The concentration of arsenic in the water supply varied from 0.01 to 1.82 mg/L, and most well-water in the endemic area had a range of 0.4–0.6 mg/L. The entire population at risk numbered 103 154. Of the people surveyed and examined clinically, characteristic arsenic-induced hyperpigmentation was diagnosed in 18.4%, keratosis in 7.1%, skin cancer in 1.1% and invasive skin cancer in 0.4%. Of the 428 people with clinically diagnosed skin cancer, 71.7% also had keratosis and 89.7% had hyperpigmentation. Ninety-nine per cent of the people with skin cancer had multiple skin cancers; 74.5% of the malignant lesions were on unexposed areas (Tseng *et al.*, 1968). Yeh (1973) studied 303 samples of skin cancers histologically: 57 were squamous-cell carcinomas, 45 were basal-cell carcinomas, 176 were intra-epidermal carcinomas (including 23 type B arsenical keratoses and 153 Bowen disease) and 25 were combined forms. The study in Taiwan lacked individual data on exposure to arsenic, since the levels were reported by village. In general, however, the incidence of hyperpigmentation, keratoses and skin cancer increased with increasing content of arsenic in the drinking-water and with age and length of exposure. The youngest patient with skin cancer was 2 years old. No case of melanosis, keratosis or skin cancer was identified in the nearby control population.

Guha Mazumder *et al*. (1998c) carried out the first population survey with individual data on exposure to arsenic among 7683 participants in West Bengal, India, to ascertain the prevalence of keratoses and hyperpigmentation. The arsenic content of their current water source ranged up to 3.4 mg/L, although 80% of participants consumed water containing < 0.5 mg/L arsenic. Of 4093 female and 3590 male participants, 48 and 108 had keratotic lesions and 127 and 234 had hyperpigmentation, respectively. Clear exposure–response relationships were found for levels of arsenic in water and the prevalence of these arsenic-induced skin effects. Men were affected more than women. Subjects who had body weights below 80% of the standard for their age and sex had a 1.6-fold and 1.2-fold increase in the prevalence of keratosis and hyperpigmentation, respectively. However, the survey examined only the participants' primary current drinking-water source. A similar cross-sectional study was conducted in Bangladesh by Tondel *et al*. (1999) who interviewed and examined 1481 subjects ≥ 30 years of age. A total of 430 subjects had skin lesions. Individual exposure assessment could only be estimated by present levels. Concentrations of arsenic in water ranged from 0.01 to 2.04 mg/L and the crude overall prevalence rate for skin lesions was 29/100. This study also showed a higher prevalence rate of arsenic-related skin lesions in men than in women, with a clear dose–response relationship.

Haque *et al*. (2003) recently completed a nested case–control study of a previous study (Guha Mazumder *et al*., 1998c) to examine the dose–response relationship between concentrations below 0.5 mg/L in drinking-water and arsenic-induced skin lesions using

a detailed exposure assessment that incorporated data on arsenic concentrations from current and past water sources used in households and work sites. A subset of 158 participants (69 cases and 89 controls) had complete histories of water concentrations. No case of a skin lesion was found with peak water concentrations of arsenic less than 0.1 mg/L. All of the eight cases (four men aged 31–75 years, four women aged 21–66 years), who currently had skin lesions and had ingested peak arsenic concentrations between 0.1 and 0.19 mg/L, had hyperpigmentation, and four also had keratoses.

Skin cancers are frequently associated with hyperkeratotic lesions (Yeh, 1973). Hyperkeratosis occurs more commonly and earlier in arsenic-exposed populations than skin cancer. A dose–response analysis of hyperkeratotic lesions may therefore allow the observation of a potential carcinogenic response to exposures lower than those used for skin cancer.

(ii) *Respiratory disease*

The possible role of chronic ingestion of arsenic in the genesis of non-malignant pulmonary disease has been suggested in a few case series describing medical problems among individuals chronically exposed to increased concentrations of arsenic in the drinking-water. Among a total cohort of 180 residents of Antofagasta, Chile, exposed to drinking-water containing 0.8 mg/L arsenic, 38.8% of 144 subjects with abnormal skin pigmentation complained of chronic cough, compared with 3% of 36 subjects with normal skin (Borgoño *et al*., 1977). In autopsies of four children and one adolescent from the Antofagasta region with an antecedent history of cutaneous arsenicosis and postmortem findings of extensive (non-pulmonary) vascular disease, two of the subjects were noted to have chronic bronchitis, slight bronchiectasis and slight diffuse interstitial fibrosis of the lung (Rosenberg, 1974). Symptoms of chronic lung disease were present in 89 (57%) of 156 cases of chronic arsenic toxicity caused by drinking arsenic-contaminated water in West Bengal, India (Guha Mazumder *et al*., 1998b). Lung function tests carried out on 17 patients showed features of restrictive lung disease in nine (53%) and combined obstructive and restrictive lung disease in seven (41%) cases.

To investigate the relationship between non-malignant respiratory disease and ingested arsenic, Guha Mazumder *et al*. (2000) analysed data from the cross-sectional survey of 7683 participants who were examined clinically and interviewed, and measured the arsenic content in their current primary drinking-water source. Arsenic concentrations ranged from < 0.003 mg/L to 3.4 mg/L. Because there were few smokers, analyses were confined to nonsmokers (n = 6864 participants). Study subjects had arsenic-associated skin lesions, such as hyperpigmentation and hyperkeratosis, and were also highly exposed at the time of the survey (concentration of arsenic in water ≥ 0.5 mg/L). Individuals with normal skin and low concentration of arsenic in water (< 0.05 mg/L) were used as the referent group. Participants with skin lesions had age-adjusted prevalence odds ratio estimates for cough, crepitations and shortness of breath of 7.8 (95% CI, 3.1–19.5), 9.6 (95% CI, 4.0–22.9) and 23.2 (95% CI, 5.8–92.8) in women and 5.0 (95% CI, 2.6–9.9), 6.9 (95% CI, 3.1–15.0) and 3.7 (95% CI, 1.3–10.6) in men, respectively.

The effect of chronic exposure to arsenic on the respiratory system was studied in 218 individuals (94 exposed to arsenic [0.136–1 mg/L] and 124 control cases), most of whom were non-smokers, in Bangladesh (Milton *et al*., 2001). The overall crude prevalence (or risk) of chronic cough and chronic bronchitis among exposed subjects was three times that in controls. Women were reported to be affected more than men.

The occurrence of chronic respiratory disease in the form of chronic cough or chronic bronchitis due to constant ingestion of arsenic through drinking-water has also been reported (Hotta, 1989; Chowdhury *et al*., 1997; Kilburn, 1997; Chakraborti *et al.*, 1998; Ahmad, S.A. *et al*., 1999; Ma *et al*., 1999; Chowdhury *et al.*, 2000b).

(iii) *Gastrointestinal system*

Chronic arsenic toxicity has been reported to produce various gastrointestinal symptoms. Hotta (1989) reported gastrointestinal impairment in 76% of subjects exposed to environmental arsenic at Torku, Japan. The symptoms were not serious in most patients, as they had possibly been afflicted with the initial stage of disease. Gastroenteritis was reported in a study of 1447 cases of chronic arsenicosis caused by drinking arsenic-contaminated water (0.05–1.8 mg/L) in the Inner Mongolian Autonomous region of China (Ma *et al*., 1999). Of patients suffering from chronic arsenicosis after drinking arsenic-contaminated water (0.05–14.2 mg/L) in West Bengal, India, gastrointestinal symptoms characterized by dyspepsia were present in 60/156 (38.4%) cases studied (Guha Mazumder *et al*., 1998b). Many investigators variously reported symptoms such as nausea, diarrhoea, anorexia and abdominal pain in cases of chronic arsenic toxicity (Rosenberg, 1974; Zaldívar, 1974; Borgoño *et al*., 1977; Cebrián *et al*., 1983; Guha Mazumder *et al*., 1988; Ahmad *et al*., 1997). However, in an epidemiological study carried out in the affected population in West Bengal, there was no difference in the incidence of abdominal pain among people drinking arsenic-contaminated water (0.05–3.4 mg/L) and the control population (< 0.05 mg/L) (27.84% versus 31.81%) (Guha Mazumder *et al*., 2001b).

(iv) *Liver and spleen*

Exposure to inorganic arsenic compounds has been associated with the development of chronic pathological changes in the liver. Several authors have reported cases of liver damage following treatment with trivalent inorganic arsenic (Morris *et al*., 1974; Cowlishaw *et al*., 1979; Szuler *et al*., 1979; Nevens *et al*., 1990). A common finding in these reports was portal hypertension without signs of liver cirrhosis. All patients had been given arsenic as a medication, mostly Fowler's solution, for several years. Typical cutaneous signs of long-term exposure to arsenic were also observed in some of the patients. In addition, there have been case reports on liver cirrhosis following medication with inorganic arsenic compounds (Franklin *et al*., 1950; Rosenberg, 1974).

Datta *et al*. (1979) reported portal hypertension associated with periportal fibrosis in nine patients who were found to have high levels of arsenic in their liver in Chandigarh, India, two of whom had been drinking arsenic-contaminated water (0.549 and 0.360 mg/L). Guha Mazumder *et al*. (1988) reported hepatomegaly in 62/67 (92.5%)

members of families who had drunk arsenic-contaminated water (0.2–2 mg/L) in West Bengal, India, but in only 6/96 (6.25%) people from the same area who had drunk uncontaminated water (< 0.05 mg/L). Thirteen arsenic-exposed patients who had hepatomegaly were further investigated in hospital. All showed varying degrees of portal zone expansion and liver fibrosis histologically. Four of the five patients who had splenomegaly showed evidence of increased intrasplenic pressure (30–36 cm saline), suggesting portal hypertension. Splenoportography of these cases showed evidence of intrahepatic portal vein obstruction. Although routine liver function tests were normal in all these cases, the bromosulphthalin retention test was abnormal in three. The level of arsenic in liver tissue, estimated by neutron activation analysis, was found to be elevated in 10/13 cases (0.5–6 mg/kg dry wt versus 0.16 ± 0.04 mg/kg dry wt in controls). Santra *et al*. (1999) and Guha Mazumder (2001a) subsequently reported hepatomegaly in 190/248 cases (76.6%) of chronic arsenicosis investigated in the same hospital. Evidence of noncirrhotic portal zone fibrosis of the liver was found histologically in 63/69 cases (91.30%) of hepatomegaly. Liver function tests carried out on 93 such patients showed evidence of elevated levels of alanine aminotransferase (ALT), aspartase aminotransferase and alkaline phosphatase in 25.8%, 61.3% and 29% of cases, respectively. Serum globulin was found to be high (> 3.5 g/dL) in 19 (20.7%) cases.

Liver enlargement has been reported in cases of chronic arsenic toxicity caused by drinking arsenic-contaminated water (Saha, 1984; Chakraborty & Saha, 1987; Ahmad, S.A. *et al*., 1997, 1999; Ma *et al*., 1999; Saha & Chakribori, 2001).

(v) *Chronic cardiovascular effects*

Ingested inorganic arsenic has been related to an increased incidence of cardiovascular disease, especially ischaemic heart disease. This has been reviewed extensively (WHO, 1981; Engel & Smith, 1994; Chen *et al*., 1997; National Research Council, 1999, 2001).

Arsenic has been well documented as one of the major risk factors for Blackfoot disease, a unique peripheral arterial disease characterized by severe systemic arteriosclerosis, as well as dry gangrene and spontaneous loss of affected extremities at end-stages. Histologically, Blackfoot disease can be divided into two reaction groups: arteriosclerosis obliterans and thromboangiitis obliterans. The prevalence of Blackfoot disease was reported to be 8.9/1000 among 40 421 inhabitants studied by Tseng *et al*. (1968) in Taiwan, China. The villages surveyed were arbitrarily divided according to the arsenic content of the well-water into low (< 0.3 mg/L), medium (0.3–0.6 mg/L) and high (> 0.6 mg/L) exposure. The prevalence of Blackfoot disease revealed a clear-cut ascendency gradient from low to medium to high exposure for both sexes and the three different age groups studied (Tseng, 1977). Atherogenicity and carcinogenicity of high levels of arsenic in artesian well-water was examined by Chen *et al*. (1988b). The lifetable method used to analyse cancer mortality of 789 patients with Blackfoot disease followed for 15 years showed a significantly higher mortality from cardiovascular and peripheral vascular disease among these patients compared with the general population in Taiwan and residents in the area endemic for Blackfoot

disease. Whether fluorescent humic substances isolated from artesian well-water play an etiological role in Blackfoot disease has not been ascertained by epidemiological or animal studies (Van Duuren *et al.*, 1986; Lu *et al.*, 1990). A causal role for arsenic in the induction of Blackfoot disease offers the best explanation for the observations in Taiwan (Engel *et al*., 1994).

Tsai *et al*. (1999) conducted a study in Taiwan, China, to analyse mortality from all causes in areas endemic for Blackfoot disease. They calculated standardized mortality ratios (SMRs) for cancer and non-cancer diseases, by sex, during the period 1971–94 and compared them with the local reference group (Chiayi-Tainan County) and the national reference group (population of Taiwan). The results revealed marked differences in SMR for the two reference groups. With respect to non-cancer disease, mortality was greater for men and women in the endemic area who had vascular disease, ischaemic heart disease, hypertension, diabetes mellitus and bronchitis than for the local reference groups. Mortality from other diseases including cancers of the rectum, stomach and oesophagus and cerebrovascular disease was higher among subjects in the study area than among the local reference group. These results indicated that the hazardous effect of arsenic was systemic.

Comparable peripheral vascular disorders with varying degrees of severity including Raynaud syndrome, acrocyanosis and gangrene of the feet have also been reported among people drinking arsenic-contaminated water (Rosenberg, 1974; Zaldívar, 1974; Borgoño *et al*., 1977; Tseng *et al*., 1996; Ahmad, S.A. *et al*., 1999; Ma *et al*., 1999; Guha Mazumder *et al*., 2001b). It should be emphasized that there are differences in the prevalence of peripheral vascular diseases that cause gangrene and limb loss among different populations exposed to arsenic; the incidence is high in Taiwan, China, but low in Chile, India and Bangladesh, and none has been reported from Mexico or Argentina (Engel *et al*., 1994).

An epidemiological study reported an increased prevalence of hypertension among residents in an area endemic for Blackfoot disease and a dose–response relationship with ingested inorganic arsenic (Chen *et al*., 1995). A total of 382 men and 516 women residing in areas of Taiwan, China, endemic for arsenic were studied. A 1.5-fold increase in the age- and sex-adjusted prevalence of hypertension was observed compared with residents in non-endemic areas, and was associated with higher cumulative exposure to arsenic. The dose–response relation remained significant after adjustment for age, sex, diabetes mellitus, proteinuria, body mass index and level of serum triglycerides. Increased prevalence of hypertension was also observed in 6.2% of patients affected with arsenic-induced skin lesions (144) compared with none of those with no skin lesion (36) in Antofagasta, Chile (Borgoño *et al*., 1977). Rahman *et al*. (1999b) conducted studies on arsenic-exposed people in Bangladesh and demonstrated an association between hypertension and cumulative exposure to arsenic in drinking-water (Rahman & Axelson, 2001; Rahman, 2002).

Significant dose–response relationships between the level of ingested inorganic arsenic and risk for ischaemic heart disease were observed in recent cohort and case–control studies in Taiwan, China (Chen *et al*., 1994). In an ecological correlational study in Taiwan based

on 898 806 person–years and 172 deaths from ischaemic heart disease observed from 1973 to 1986, a dose–response relationship between concentration of arsenic in drinking-water and age-adjusted mortality from ischaemic heart disease was observed. A total of 257 patients with Blackfoot disease and 753 matched healthy controls were recruited and followed-up for more than 7 years. Significantly increased mortality from ischaemic heart disease was observed for patients with Blackfoot disease and matched controls showing SMRs (95% confidence interval [CI]) of 937 (536–1519) and 248 (139–409), respectively, compared with the general population of Taiwan (SMR, 100). Cox's proportional hazard regression analysis also showed a dose–response relationship between mortality from ischaemic heart disease and cumulative exposure to arsenic after adjustment for age, sex, body mass index and disease status for hypertension and diabetes mellitus. A case–control study including 78 patients with electrocardiogram-based ischaemic heart disease and 384 healthy residents was carried out in three villages where Blackfoot disease was endemic. Based on a multiple logistic regression analysis, cumulative exposure to arsenic was found to be associated with ischaemic heart disease in a dose-related manner after adjustment for age, sex, body mass index, disease status for hypertension and diabetes mellitus, ratio between total cholesterol and high-density lipoprotein cholesterol and cumulative alcohol consumption. The occurrence of ischaemic heart disease due to chronic exposure to arsenic has also been reported by other investigators (Rosenberg, 1974; Zaldívar, 1974; Hotta, 1989; Chen *et al.*, 1994, 1995; Ma *et al*., 1999), as has the occurrence of cardiac arrhythmia (Hotta, 1989; Ma *et al*., 1999).

Mortality rates from 1973 through 1986 for ischaemic heart disease among residents in 60 villages of an area in Taiwan, China, endemic for arsenicosis were analysed by Chen *et al*. (1996) to examine their association with the concentration of arsenic in drinking-water. Based on 1 355 915 person–years and 217 deaths from ischaemic heart disease, the cumulative mortality from birth to age 79 years as 3.4%, 3.5%, 4.7% and 6.6%, respectively, for residents who lived in villages in which the median concentrations of arsenic in drinking-water were < 0.1, 0.1–0.34, 0.35–0.59 and ≥ 0.6 mg/L. A cohort of 263 patients with Blackfoot disease and 2293 residents in the endemic area of arsenicosis without Blackfoot disease were recruited and followed up for an average period of 5.0 years. There was a monotonic biological gradient relationship between cumulative exposure to arsenic through drinking artesian well-water and mortality from ischaemic heart disease. The relative risks (95% CI) were 2.5 (0.53–11.37), 4.0 (1.01–15.60) and 6.5 (1.88–22.24), respectively, for those who had cumulative exposures to arsenic of 0.1–9.9, 10.0–19.9 and ≥ 20.0 mg/L–years, compared with those with no known exposure to arsenic, after adjustment for age, sex, cigarette smoking, body mass index, serum cholesterol and triglyceride levels, and disease status for hypertension and diabetes through proportional hazard regression analysis. Patients with Blackfoot disease were found to have a significantly higher mortality from ischaemic heart disease than residents without Blackfoot disease, showing a multivariate-adjusted relative risk of 2.5 (95% CI, 1.14–5.40).

Wang *et al*. (2002) reported evidence of a dose–response relationship between long-term exposure to arsenic in drinking-water and prevalence of carotid atherosclerosis in the

arsenic-exposed area of south-western Taiwan, China. The extent of carotid atherosclerosis was assessed by duplex ultrasonography among 199 male cases and 264 residents who participated in the study. Three indices of exposure, duration of consumption of artesian well-water, average concentration of arsenic in consumed well-water and cumulative exposure to arsenic, were all significantly associated with an increased prevalence of carotid atherosclerosis with a dose–response relationship. The biological gradient remained significant after adjustment for age, sex, hypertension, diabetes mellitus, cigarette smoking, alcohol consumption, waist-to-hip ratio and serum levels of total and low-density lipoprotein cholesterol. The multivariate-adjusted prevalence odds ratio was 1.8 (95% CI, 0.8–3.8) and 3.1 (95% CI, 1.3–3.4) for those who had a cumulative exposure to arsenic of 0.1–19.9 and ≥ 20 mg/L–years, respectively, compared with those without exposure to arsenic from drinking artesian well-water.

(vi) *Nervous system*

Abnormal electromyographic (EMG) findings suggestive mostly of sensory neuropathy were reported in 10/32 (31.25%) subjects exposed to arsenic by drinking contaminated well-water (range, 0.06–1.4 mg/L) in Canada (Hindmarsh *et al*., 1977). Paresthaesia was present in 74/156 (47.43%) patients with chronic arsenicosis caused by drinking arsenic-contaminated water (0.05–14.2 mg/L) in West Bengal, India. Objective evaluation of neuronal involvement carried out in 29 patients showed abnormal EMGs in 10 (34.5%) and altered nerve conduction velocity and EMGs in 11 (38%) cases (Guha Mazumder *et al*., 1997). Evidence of parasthaesia or peripheral neuropathy due to chronic exposure to arsenic through drinking-water has also been reported (Saha, 1984; Hotta, 1989; Kilburn, 1997; Ahmad, S.A. *et al*., 1999; Ma *et al*., 1999; Chowdhury *et al*., 2000a; Rahman *et al*., 2001, 2003). More sensory than motor neuropathy has also been reported among arsenicosis patients in West Bengal (Basu *et al*., 1996; Mukherjee *et al*., 2003).

The relationship between the prevalence of cerebrovascular disease and ingestion of inorganic arsenic in drinking-water was reported by Chiou *et al*. (1997a) in a cross-sectional study in Taiwan, China, that recruited a total of 8102 men and women from 3901 households. The status of cerebrovascular disease of study subjects was identified through personal home interviews and ascertained by review of hospital medical records according to WHO criteria. Information on consumption of well-water, sociodemographic characteristics, cigarette smoking habits and alcohol consumption, as well as personal and family history of disease, was also obtained. The concentration of arsenic in the well-water of each household was determined by HG–AAS. A significant dose–response relationship was observed between concentration of arsenic in well-water and the prevalence of cerebrovascular disease after adjustement for age, sex, hypertension, diabetes mellitus, cigarette smoking and alcohol consumption. The biological gradient was even more prominent for cerebral infarction, showing multivariate-adjusted odds ratios of 1.0, 3.21 (95% CI, 1.51–6.88), 4.37 (95% CI, 1.99–9.60) and 6.58 (95% CI, 2.82–15.28), respectively, for those who consumed well-water with an arsenic content of 0, 0.001–0.05, 0.051–0.299 and

> 0.3 mg/L. Increased incidences of cerebrovascular disease in cases of chronic arsenicosis have been reported elsewhere (Hotta, 1989; Chen *et al.*, 1997; Ma *et al*., 1999).

Kilburn (1997) reported the occurrence of peripheral neuritis, sleep disturbances, weakness and cognitive and memory impairment in residents of Bryan-College Station, TX, USA, exposed to arsenic in air and water from the use of arsenic trioxide to produce defoliants for cotton at an Atochem plant. Siripitayakunkit *et al*. (1999) reported retardation of intelligence among 529 children (6–9 years of age) living in Thailand who had chronic exposure to arsenic from the environment.

(vii) *Diabetes mellitus*

To examine the association between ingested inorganic arsenic and the prevalence of diabetes mellitus, Lai *et al*. (1994) studied 891 adults residing in villages in southern Taiwan, China. The status of diabetes mellitus was determined by an oral glucose tolerance test and a history of diabetes regularly treated with sulfonylureas or insulin. They observed a dose–response relationship between cumulative exposure to arsenic and prevalence of diabetes mellitus. The relationship remained significant after adjustment for age, sex, body mass index and physical activity level at work by a multiple logistic regression analysis, giving multivariate-adjusted odds ratios of 6.61 (95% CI, 0.86–51.0) and 10.05 (95% CI, 1.30–77.9), respectively, for those who had a cumulative exposure to arsenic of 0.1–15.0 and > 15.1 mg/L–years compared with those who were unexposed.

Rahman *et al.* (1998) reported a significantly increased prevalence of diabetes mellitus in Bangladesh caused by drinking arsenic-contaminated water among subjects with keratosis compared with subjects who did not have keratosis. A significant trend in risk between an approximate, time-weighted exposure to arsenic and the prevalence of diabetes mellitus strengthened the possibility of a causal association. [The lack of comprehensive, systematic, long-term sampling of the water supplies in the study area is a limitation of the study and data on individual exposures measured directly over time would have been more informative. However, these results suggest that chronic exposure to arsenic may induce diabetes mellitus in humans.] A further study regarding glucosuria patients with and without skin lesions in relation to exposure to arsenic in drinking-water reported that the prevalence ratios among the subjects without skin lesions were 0.8 (95% CI, 0.4–1.3), 1.4 (95% CI, 0.8–2.3) and 1.4 (95% CI, 0.7–2.4), after ajustment for age and sex compared with unexposed subjects as reference. The exposure categories were < 0.5, 0.5–1 and > 1 mg/L, respectively. For those with skin lesions, the prevalence ratios were slightly higher; 1.1 (95% CI, 0.5–2.0), 2.2 (95% CI, 1.3–3.8) and 2.6 (95% CI, 1.5–4.6), respectively, in comparison with unexposed subjects (Rahman *et al.*, 1999a). [In this study also, a lack of systematic sampling of water supplies in the study area is a limitation. Furthermore, although glucosuria is a primary indicator of diabetes mellitus, identification of the hyperglycaemic patients among those with glucosuria would have been more informative.]

Tseng *et al*. (2000) reported a cohort study on 446 non-diabetic residents from an arsenic-contaminated area in south-western Taiwan, China. Diabetes mellitus was deter-

mined by an oral glucose tolerance test. The age-specific incidence density ratio of diabetes mellitus was between two- and five-fold higher in the exposed cohort than in the unexposed cohort. An exposure–response relationship was observed between incidence of diabetes mellitus and long-term exposure to ingested arsenic from artesian well-water, showing a relative risk of 2.1 (95% CI, 1.1–4.2) for those who had a cumulative exposure to arsenic ≥ 17 mg/L–years compared with those who had a lower cumulative exposure (< 17 mg/L–years).

(viii) *Study of oxidative stress in humans*

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is generated by the hydroxyl radical (Kasai & Nishimura, 1984) or singlet oxygen (Devasagayam *et al*., 1991) or by direct electron transfer, which does not involve the participation of any reactive oxygen species (Kasai *et al*., 1992). 8-OHdG is considered to be one of the main indicators of oxidative damage to DNA and may cause mutation (G:C→T:A) during DNA replication (Shibutani *et al*., 1991).

Matsui *et al*. (1999) investigated whether neoplastic and precancerous skin lesions of arsenic-exposed individuals are under oxidative stress using 8-OHdG as a marker. Biopsy samples of arsenic keratosis, arsenic-induced Bowen disease and arsenic-induced Bowen carcinoma arising in areas not exposed or less exposed to the sun were obtained from 28 individuals (aged 26–83 years) living in areas where chronic arsenicism was endemic in either Taiwan, China, Thailand or Japan. The presence of 8-OHdG was studied by immunohistochemistry using N45.1 monoclonal antibody in the 28 cases of arsenic-related skin neoplasm and arsenic keratosis as well as in 11 cases of Bowen disease unrelated to arsenic. The frequency of 8-OHdG-positive cases was significantly higher in arsenic-related skin neoplasms (22/28; 78%) than in Bowen disease unrelated to arsenic (1/11; 9%) ($p < 0.001$ by chi-square test). 8-OHdG was also detected in normal tissue adjacent to the arsenic-related Bowen disease lesion. Furthermore, arsenic was detected by neutron activation analysis in deparaffined skin tumour samples of arsenic-related disease (four of five, 80%), whereas it was not detected in control samples. The results may suggest the involvement of reactive oxygen species in arsenic-induced human skin cancer.

Wu *et al*. (2001) reported an association of blood arsenic levels with increased reactive oxidants and decreased antioxidant capacity among 64 subjects aged 42–75 years from an arsenic-contaminated area in north-eastern Taiwan, China. The blood level of arsenic determined by HG–AAS ranged from undetectable to 46.5 μg/L. The capacity of subjects to methylate arsenic was determined by speciation of inorganic arsenic and its metabolites in urine using high-performance liquid chromatography (HPLC) linked with HG–AAS. The plasma level of reactive oxidants was determined by a chemiluminescence method using lucigenin as an amplifier for measuring superoxide anion (O_2^-), while the plasma level of antioxidant capacity was measured by the 2,2′-azino-di[3-ethylbenzthiazoline]-sulfonate method. There was a positive association between blood arsenic level and plasma level of reactive oxidants ($r = 0.41$; $p = 0.001$) and a negative association with plasma level of antioxidant capacity ($r = –0.30$; $p = 0.014$). Categorical analysis showed that greater

primary capacity for arsenic methylation was correlated with a higher plasma level of antioxidant capacity ($p = 0.029$).

(ix) *Other*

Generalized weakness and fatigue have been reported in people chronically exposed to arsenic-contaminated drinking-water (Zaldívar & Guillier, 1977; Saha, 1984; Guha Mazumder *et al*., 1988, 1992; Kilburn, 1997; Guha Mazumder *et al*., 1998; Guha Mazumder, 2001b; Guha Mazumder *et al.*, 2001b). Conjunctival congestion and non-pitting oedema of the legs and hands have also been reported in patients with chronic arsenic toxicity in West Bengal, India, and Bangladesh (Ahmad *et al*., 1997; Chowdhury *et al*., 1997; Guha Mazumder *et al*., 1998b; Ahmad, S.A. *et al*., 1999).

García Vargas *et al*. (1994) carried out a detailed study of the urinary excretion pattern of porphyrins in humans chronically exposed to arsenic via drinking-water in Mexico using HPLC. Thirty-six individuals (15 men and 21 women) were selected from a town which had 0.40 mg/L arsenic in the drinking-water. The control group consisted of 31 individuals (13 men and 18 women) whose concentration of arsenic in the drinking-water was 0.02 mg/L. Major abnormalities in the urinary porphyrin excretion pattern observed in arsenic-exposed individuals were (*a*) significant reductions in coproporphyrin III excretion resulting in decreases in the coproporphyrin III/coproporphyrin I ratio and (*b*) significant increases in uroporphyrin excretion. Both alterations were responsible for the decrease in the urinary coproporphyrin/uroporphyrin ratio. No porphyrinogenic response was found in individuals with urinary concentrations below 1 mg arsenic/g creatinine. However, as arsenic concentrations exceeded this value, the excretion of porphyrins (except coproporphyrin III) increased proportionally, and most of the individuals with high urinary arsenic concentrations had alterations in porphyrin ratios and also presented cutaneous signs of chronic arsenic poisoning. The prevalence of clinical signs of arsenicism showed a direct relationship with both concentration of arsenic in urine and time-weighted exposure to arsenic. A direct relationship between time-weighted exposure to arsenic and alterations in urinary porphyrin excretion ratios was also observed. These alterations in arsenic-exposed individuals are compatible with a lower activity of uroporphyrinogen decarboxylase, the enzyme that converts the substrate uroporphyrinogen to a coproporphyrinogen product.

Except for anaemia, no haematological abnormality (in differential lymphocyte count or in the levels of blood sugar, urea or creatinine) has been described in cases of chronic toxicity caused by drinking arsenic-contaminated water (Guha Mazumder *et al*., 1988, 1997, 1998b, 1999). Haematological consequences of subacute and chronic arsenic toxicity have been reviewed extensively (National Research Council, 1999).

4.2.2 *Experimental systems*

(*a*) *Acute toxicity*

The acute toxicity of arsenic is related to its chemical form and oxidation state. The LD_{50} (50% lethal dose) values of several arsenicals in laboratory animals have been

reviewed (Hughes, 2002). In mice, the oral lethal dose of arsenic trioxide varies from 15 to 48 mg/kg bw. In contrast, the lethal dose range of inorganic arsenic in adult humans is estimated at 1–3 mg/kg bw (Hughes, 2002). A basic tenet is that the acute toxicity of trivalent arsenic is greater than that of pentavalent arsenic. For example, in mice, the oral LD_{50} of arsenic trioxide is more than 36-fold lower than that of MMA^{V}. However, MMA^{III} has been found to be more toxic than trivalent arsenic. When MMA^{III} or sodium arsenite was administered intraperitoneally to hamsters, the LD_{50}s were found to be 29.3 and 112.0 μmol/kg bw, respectively (Petrick *et al*., 2001).

(*b*) *Chronic toxicity*

Many different systems within the body are affected by chronic exposure to inorganic arsenic. Arsenates can replace phosphate in many biochemical reactions because they have similar structure and properties. Arsenate uncouples in-vitro formation of adenosine-5′-triphosphate (ATP) by a mechanism termed arsenolysis. In the substrate, arsenolysis may occur during glycolysis. ATP is generated during glycolysis in the presence of phosphate (substrate phosphorylation), but not arsenate. In the mitochondria, arsenolysis may occur during oxidative phosphorylation. Adenosine-5′-diphosphate (ADP)-arsenate is synthesized by submitochondrial particles from ADP and arsenate, in the presence of succinate. ADP-arsenate hydrolyses easily compared with ADP-phosphate, which is formed during oxidative phosphorylation. In both the substrate and the mitochondria, arsenolysis diminishes in-vitro formation of ATP by the replacement of phosphate with arsenate in the enzymatic reactions. Depletion of ATP by arsenate has been observed in cellular systems: ATP levels are reduced in rabbit and human erythrocytes after in-vitro exposure to arsenate (rabbits, 0.8 mM; humans, 0.01–10 mM) (Delnomdedieu *et al.*, 1994a; Winski & Carter, 1998; Hughes, 2002).

Trivalent arsenic reacts readily *in vitro* with thiol-containing molecules such as GSH and cysteine (Scott *et al.*, 1993; Delnomdedieu *et al.*, 1994b). In rat red blood cells, As^{III} forms mixed complexes with protein and GSH, and the main protein-binding species is haemoglobin (Winski & Carter, 1995). Binding of MMA^{III} and DMA^{III} to protein *in vitro* occurs to a greater extent than with the pentavalent organic forms (Styblo *et al.*, 1995). Arsenite has a higher affinity for dithiols than monothiols, as shown by the highly favoured transfer of arsenite from a $(GSH)_3$–arsenic complex to the dithiol 2,3-dimercaptosuccinic acid (Delnomdedieu *et al.*, 1993). The binding of trivalent arsenic to critical thiol groups may inhibit important biochemical events that could lead to toxicity (Hughes, 2002).

(i) *Mitochondrial damage*

Hepatic phosphate resonances were evaluated by Chen *et al*. (1986) *in vivo* by ^{31}P nuclear magnetic resonance spectroscopy following a single intravenous dose of sodium arsenite (10 mg/kg bw) in rats. Acute in-vivo administration of arsenite rapidly decreased intracellular pools of ATP with concomitant increases in inorganic phosphate and phosphomonoesters (phosphocholine and adenosine monophosphate). Glycerolphosphorylcholine and glycerolphosphorylethanolamine were also increased. The data suggest

that liver cannot compensate for the rapid loss of nicotinamide-adenine dinucleotide (NAD)-linked substrate oxidation via other metabolic pathways, such as glycolysis, for the production of ATP.

Arsenic fed to laboratory animals is known to accumulate in the mitochondria and has been related to the swelling of this subcellular organelle in a number of tissues, especially the liver (Fowler *et al.*, 1977, 1979). It has been suggested that the effects of arsenic on mitochondrial utilization of pyruvate results from arsenic binding to the lipoic acid and dithiol moieties of the pyruvate dehydrogenase (PDH) complex. The initial step in the mitochondrial metabolism of pyruvate, which is catalysed by the PDH–enzyme complex, involves the formation of acetyl-coenzyme A (CoA) and the generation of CO_2 and hydrogenated NAD (NADH). This complex is composed of three enzymes: pyruvate decarboxylase (PDH), dehydrolipoate transacetylase and dihydrolipoate dehydrogenase. The latter two enzymes contain dithiol moieties. Pyruvate decarboxylase is regulated by inactivation and activation reactions, which are controlled by phosphorylation/dephosphorylation. Phosphorylation and the concomitant inactivation of PDH is catalysed by a Mg-ATP-requiring kinase, and dephosphorylation and concomitant reactivation is catalysed by a Mg^{2+}- and Ca^{2+}-requiring phosphatase. In order to examine whether this phosphorylation/dephosphorylation is a mechanism of action for arsenic, PDH activities, before and after in-vitro activation with Mg^{2+}, were measured in tissue from animals fed arsenic (Schiller *et al*., 1977). Adult male Charles River CD rats were given deionized drinking-water containing 0, 20, 40, and 85 mg/L arsenic as sodium arsenate (As^V) for 3 and 6 weeks. PDH activity was assayed in the liver tissue obtained from the animals. After 3 weeks, the effects of arsenic at the highest dose were pronounced compared with the basal activity (before activation), with up to 47.5% inhibition of the control values. The total PDH activity (after activation) was inhibited by 13.5, 15.3 and 27.6% of the control values at 20, 40 and 85 mg/L sodium arsenate, respectively. A similar pattern of inhibition of PDH activity was observed at 6 weeks, although the inhibition was lower at the highest dose. This pattern may be indicative of mitochondrial regeneration at the highest dose after 6 weeks (Schiller *et al.*, 1977). The possible metabolic effects of this inhibition are a decrease in acetyl-CoA formation, which leads to a decrease in carbon flow through the tricarboxylic acid cycle, and a decrease in the citrate available to allow mitochondria to supply acetyl-CoA for fatty acid synthesis, which in turn results in fewer storage triglycerides.

Petrick *et al*. (2001) have compared the in-vivo toxicity of MMA^{III} and arsenite in hamsters. Groups of six male golden Syrian hamsters, 11–12 weeks old and weighing 100–130 g, were injected intraperitoneally with MMA^{III} oxide or sodium arsenite. Inhibition of PDH activity of the hamster kidney or purified porcine heart by MMA^{III} or arsenite was determined. To inhibit PDH activity of hamster kidney by 50%, concentrations (mean ± SE) of 59.9 ± 6.5 μM MMA^{III} as methylarsine oxide, 62.0 ± 1.8 μM MMA^{III} as diiodomethylarsine and 115.7 ± 2.3 μM arsenite were needed. To inhibit in-vitro PDH activity of the purified porcine heart by 50%, concentrations (mean ± SE) of 17.6 ± 4.1 μM MMA^{III} as methylarsine oxide and 106.1 ± 19.8 μM arsenite were needed. These data demonstrate that MMA^{III} is more toxic than inorganic arsenite, both *in vivo* and *in vitro*.

Brown *et al*. (1976) demonstrated alteration of normal ultrastructure and respiratory ability of proximal renal tubules following administration of arsenate. Groups of male Sprague-Dawley rats weighing 70–150 g were fed laboratory chow. The control groups received deionized water while the experimental groups received 40, 85 or 125 mg/L arsenic as sodium arsenate in deionized water (n = 28, 11, 7 and 10 in each group, respectively). After 6 weeks, the rats were killed in pairs of one experimental rat and a control rat matched by weight. The kidneys were excised and the capsule removed; combined oxygen electrode and electron microscopic studies were conducted. Decreased state 3 respiration and respiratory control ratios were observed in kidneys of rats given the 85- and 125-mg/L dose levels. Ultrastructural alterations, which consisted of swollen mitochondria and an increased number of dense autophagic lysosome-like bodies, were confined to proximal tubule cells of animals at all dose levels of arsenic.

Fowler *et al*. (1977) carried out investigations to delineate the subcellular manifestations of arsenic toxicity following chronic exposure using combined ultrastructural and biochemical techniques. Four groups of 18 male Charles River CD rats were fed a casein-based purified diet and had access to deionized drinking-water containing 0, 20, 40 or 85 mg/L arsenic as sodium arsenate (As^{v}) for 6 weeks. At the end of this period, three animals from each group were killed and the livers removed. Mitochondrial respiration studies were conducted. Extensive in-situ swelling of liver mitochondria and matrix rarification with lipidic vacuolation were the most prominent ultrastructural changes observed at 40- and 80-mg/L As^{V} dose levels. Mitochondrial respiration studies indicated decreased state 3 respiration and respiratory control ratios for pyruvate/malate- but not succinate-coupled respiration. Specific activity of monoamine oxidase, which is localized on the outer mitochondrial membrane, showed increases of up to 150% of control, and cytochrome *c* oxidase, which is localized on the inner mitochondrial membrane, showed an increase in specific activity of 150–200%. Activity of malate dehydrogenase, which is localized in the mitochondrial matrix, remained unchanged at all dose levels. These studies indicate that decreased mitochondrial respiration is only one aspect of arsenic toxicity to this organelle. Marked arsenic-mediated perturbation of important enzyme systems localized in mitochondria, which participate in the control of respiration and other normal mitochondrial functions (such as haeme synthesis, carbohydrate metabolism and fatty acid synthesis), are also important manifestations of cellular dysfunction.

A positive, quantitative in-vivo correlation between mitochondrial structure and function and their alteration following administration of sodium arsenate has further been demonstrated (Fowler *et al*., 1979). Two groups of male Charles River CD rats were fed a casein-based semipurified diet for 6 weeks and had access to deionized drinking-water containing 0 or 40 mg/L arsenic as sodium arsenate. Ultrastructural morphometric and biochemical studies were conducted on hepatic mitochondria. Morphometric analysis disclosed an overall 1.2-fold increase in the relative mitochondrial volume density and a 1.4-fold increase in the surface density of the inner mitochondrial membrane plus cristae of arsenate-exposed rats. These observations suggest that arsenate-mediated perturbation of mitochondrial membrane integrity compromises the mechanisms of normal ion

transport. These structural changes were associated with a perturbation of mitochondrial protein synthesis as expressed by a 1.5-fold increase in [^{14}C]leucine incorporation into all mitochondrial proteins, which was primarily associated with the acid-insoluble membranous fraction. Mitochondria from arsenate-treated rats showed a marked disruption of normal conformational behaviour with depression of NAD^+-linked substrate oxidation and a subsequent approximately two-fold in-vivo increase in the mitochondrial NAD^+ to $NADH^+$ ratio. Observed changes in mitochondrial membranes from arsenate exposure also resulted in 1.5–2-fold increases in the specific activities of the membrane marker enzymes monoamine oxidase, cytochrome c oxidase and Mg^{2+}-ATPase, which are localized in both inner and outer mitochondrial membranes. Activity of malate dehydrogenase, which is localized in the mitochondrial matrix, was unchanged.

Larochette *et al*. (1999) investigated whether arsenic compounds act on mitochondria to induce apoptosis. The mechanisms by which arsenic induces apoptosis are not clear. U937 cells transfected with an SFFV.neo-vector containing the human *bcl-2* gene coding for apoptosis-inhibitory protein or the neomycin-resistance gene (*Neo*) only, or 2B4.11T cell hybridoma cells ($1–5 \times 10^5$/mL) were incubated with variable doses of sodium arsenite, sodium arsenate, phenylarsine oxide, *para*-arsanilic acid or 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) and 100 μM of the caspase inhibitors *N*-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk) and *tert*-butyloxycarbonyl-Asp.fluoromethylketone (Boc-D.fmk) and 100 μM of the cathepsin inhibitor *N*-benzyloxycarbonyl-Phe-Ala.fluoromethylketone (Z-FA.fmk). Arsenite induced apoptosis accompanied by a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Inhibition of caspases by Z-VAD.fmk and Boc-D.fmk prevented arsenite-induced nuclear DNA loss, but had no effect on the $\Delta\Psi_m$ dissipation and cytolysis induced by arsenite, suggesting that arsenite might cause necrosis when the caspase pathway is blocked. In contrast, *bcl-2* expression induced by gene transfer prevented all hallmarks of arsenite-induced cell death (such as generation of reactive oxygen species, hypoploidy and loss of viability), including the collapse of $\Delta\Psi_m$. PK11195, a ligand of the mitochondrial benzodiazepine receptor, neutralized the bcl-2-mediated resistance to arsenite. Mitochondria were required in a cell-free system (isolated nuclei *in vitro*) to mediate arsenite-induced nuclear apoptosis. Arsenite caused the release of an apoptosis-inducing factor from the mitochondrial intermembrane space. This effect was prevented by the permeability transition (PT) pore inhibitor cyclosporin A, as well as by bcl-2, which is known to function as an endogenous PT pore antagonist. Arsenite-permeabilized liposomal membranes contained the purified, reconstituted PT pore complex. Bcl-2 also inhibited the arsenite-triggered opening of the PT pore in the reconstituted system. As a control, a mutant bcl-2 $\Delta\alpha$5/6 protein, which had lost its anti-apoptotic function as a result of the deletion of a putative membrane insertion domain, failed to prevent arsenite-induced PT pore opening. Together these data suggest that arsenite could induce apoptosis via a direct effect on the mitochondrial PT pore.

(ii) *Urinary porphyrins, haeme biosynthetic enzyme activities, haeme metabolism and arsenic*

Arsenic can modify the urinary excretion of porphyrins in animals and humans. It also interferes with the activities of several enzymes of the haeme biosynthetic pathway, such as δ-aminolevulinate (ALA) synthase (ALA-S), porphobilinogen deaminase, uroporphyrinogen III synthase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, ferrochelatase and haeme oxygenase (H-O). The urinary porphyrins and several haeme enzymes can be used as early biomarkers of arsenic toxicity (García-Vargas & Hernández-Zavala, 1996).

Rodents exposed for 6 weeks to sodium arsenate in drinking-water showed a substantial increase in the urinary excretion of porphyrins, with excretion of uroporphyrin exceeding that of coproporphyrin (Woods & Fowler, 1978). Groups of 12 male Sprague-Dawley rats (CD strain) (150–200 g) or male C57 BL mice (20–30 g) were given access to laboratory chow and deionized drinking-water containing 0, 20, 40 or 85 mg/L arsenic as sodium arsenate (As^{v}) for up to 6 weeks. Livers of animals were homogenized and mitochondria and microsomal fractions were then prepared. Continuous prolonged exposure to sodium arsenate resulted in depression of hepatic δ-aminolevulinate synthase and haeme synthase, the first and last enzymes in haeme biosynthesis, respectively, in both rats and mice. ALA-S was maximally depressed to approximately 80% of control values at 40 mg/L in both species, whereas haeme synthase activity was maximally decreased to 63 and 75% of control at 85 mg/L in rats and mice, respectively. Uroporphyrinogen I synthase, the third enzyme in haeme biosynthesis, was increased at all doses in mice, whereas ALA dehydratase, the second haeme biosynthetic pathway enzyme, was unaltered in either species. Concomitantly, urinary uroporphyrin concentrations were increased up to 12-fold and coproporphyrin levels up to 9-fold the control values in rats. Similar patterns of increased porphyrin excretion were seen in mice. In contrast, no changes were observed in the activities of cytochrome oxidase or cytochrome P450, indicators of mitochondrial and microsomal haemoprotein function, respectively. These results demonstrate that prolonged exposure to low levels of arsenic results in selective alteration of hepatic haeme biosynthetic pathway enzymes, with concomitant increases in urinary porphyrin concentrations.

Cebrián *et al*. (1988) demonstrated that sodium arsenite is a potent inducer of H-O, which is the rate-limiting enzyme of haeme degradation. Male Wistar albino rats were fasted for 24 h before treatment and until they were killed. Animals received 0.1 mL sodium chloride (0.9%, w/v), or arsenic salts by subcutaneous injection. The doses of As^{III} were 12.5, 25, 50, 75 and 100 μmol/kg bw and those of As^{V} were 25, 50, 100, 150 and 200 μmol/kg bw. Animals were killed 16 h after injection. In a subchronic study, animals were exposed to As^{III} in the drinking-water at a concentration of 50 mg/L for periods of 5, 10, 20 or 30 days, and food was withheld for 24 h before sacrifice. The livers were excised, perfused and homogenized, and tryptophan pyrrolase (TP), ALA-S and H-O activities were measured. Cytochrome P450 and *b5* contents were also measured. Acute administration of arsenic produced a decrease in the haeme saturation of TP in rat liver,

accompanied by dose-related increased ALA-S and H-O activities, and a corresponding decrease in cytochrome P450 concentration. The decrease in the haeme saturation of TP indicates that arsenic reduced the content of cytosolic haeme in liver cells and that the increase in hepatic ALA-S activity appears to be in response to a reduction in haeme availability. The alteration in the relationship between haeme synthesis and degradation is a result of treatment with arsenic. The magnitude of these effects was related to the oxidation state of arsenic: sodium arsenite (As^{III}) was more potent than sodium arsenate (As^{V}). These results support the suggestion that haeme saturation of TP is sensitive to treatments that modify liver haeme concentration. The increase in H-O activity produced by arsenic appears to be mediated by a mechanism largely or entirely independent of haeme. Indeed, there were no indications of an increase in the free haeme pool that could trigger a positive feedback on H-O. On the contrary, it appears that one reason for the reduction in haeme saturation was the increase in H-O activity. Moreover, the concomitant increase in ALA-S activity was a further indication of cellular depletion of haeme. The main effects of continuous exposure to As^{III} were an initial decrease in the haeme saturation of TP, which remained constant during the period of treatment, and an initial increase in ALA-S activity, which after 10 days of exposure dropped somewhat but remained above control values. No significant effects on H-O or P450 activity were observed. These results were interpreted as being indicative that a new balance between haeme synthesis and degradation had been reached and that an adaptive response to the subchronic effects of As^{III} was taking place.

(iii) *Arsenic and oxidative stress*

Among the various proposed mechanisms by which arsenic induces cancer, oxidative damage may play a role in arsenic-induced carcinogenesis. Exposure to arsenite, arsenic trioxide or arsenate has been reported to result in the generation of reactive oxygen species in laboratory animals or in cultured animal and human cells by many investigators (Wang *et al*., 1996; Chen *et al*., 1998; Hei *et al*., 1998; Ahmad *et al*., 2000; Lynn *et al*., 2000; Chouchane & Snow, 2001; Liu, S.X. *et al*., 2001). The topic has been reviewed by Del Razo *et al*. (2001b), Ercal *et al*. (2001), and Thomas *et al*. (2001).

Arsenic-induced free-radical formation was indicated by Yamanaka *et al*. (1991), who studied cellular response in the lung induced by the administration of DMA^{V}, and in particular the enzymes that participate directly in protective reactions against active oxygen species, superoxide dismutase, catalase and GSH peroxidase (GPx). Male ICR mice, weighing approximately 25 g, were given an oral dose of 1500 mg/kg bw DMA^{V} after fasting for several hours. The activities of mitochondrial superoxide dismutase, GPx and glucose-6-phosphate dehydrogenase (G6PDH) significantly increased at 6 h or longer after dosing, whereas cytosolic superoxide dismutase and catalase were not. Furthermore, the NADPH levels were markedly decreased at 6–9 h after treatment with DMA^{V} while $NADP^{+}$ levels increased, resulting in a marked reduction in the NADPH/$NADP^{+}$ ratio. This change, accompanied by an increase in G6PDH activity, indicates that the pentose-phosphate pathway is activated by the oxidation of reduced GSH with hydrogen peroxide

(H_2O_2). With regard to cellular sulfhydryls, after treatment with DMA^V, levels of GSH and non-protein sulfhydryls were decreased and levels of oxidized GSH (GSSG) remained constant, whereas those of mixed disulfides were significantly increased. These cellular variations suggest that mouse pulmonary cells produced reduced oxygen species, that is, superoxide anion radical, hydrogen peroxide and subsequent radicals in the metabolism of DMA^V, and that these and the dimethylarsenic peroxyl radical were responsible for pulmonary DNA damage, the diethylarsenic peroxyl radical probably being produced from the reaction of molecular oxygene and dimethylarsine (Yamanaka *et al.*, 1990, 2001). The same investigators had demonstrated previously that oral administration of DMA^V, the main metabolite of inorganic arsenic, induces lung-specific DNA damage in mice. An in-vitro experiment indicated that the breaks were not caused directly by DMA^V but by DMA^{III}, a further metabolite of DMA. They hypothesized that this damage was partially due to the active oxygen species produced in the metabolism of DMA^V (Yamanaka *et al.*, 1989). In a further study, the authors had shown that oral administration of DMA to mice significantly enhanced the amounts of 8-oxo-2′-deoxyguanosine (8-oxodG) specifically in target organs of arsenic carcinogenesis (skin, lung, liver and urinary bladder) and in urine. The dimethyl arsenics thus may play an important role in the carcinogenesis of arsenic through the induction of oxidative damage, particularly of base-oxidation (Yamanaka *et al.*, 2001).

Ahmad, S. *et al*. (1999) investigated the biochemical effects of exposure to DMA in $B6C3F_1$ mice using six biochemical parameters: DNA damage, GSH and GSSG content, cytochrome P450 content, ornithine decarboxylase (ODC) activity in liver and/or lung and ALT activity in serum. GSH was selected as an important constituent for cellular protection against oxidative damage by free radicals and the three enzymes were employed as biological markers of cell proliferation and promotion of carcinogenesis. Groups of 10 or 12 adult female $B6C3F_1$ mice received DMA^V at a dose of 720 mg/kg bw by oral gavage at one of three times (2 h, 15 h or at both 21 and 24 h) before sacrifice. Four or five control mice were run on each of 5 experimental days and received distilled water alone. Significant ($p < 0.05$) decreases in liver GSH and GSSG contents (15–37%) were observed. Pulmonary and hepatic ODC activities were reduced (19–59%) by treatment with DMA^V. A significant decrease in hepatic cytochrome P450 content (21%) was observed only in the group treated at both 21 and 24 h before sacrifice. The mouse serum ALT activity was not reduced after in-vivo administration of DMA^V but the addition of 2.8, 28 and 280 mM DMA^V *in vitro* reduced ALT activity by 0, 8 and 6.5%, respectively.

Santra *et al*. (2000) examined the hepatic effects of chronic ingestion (for up to 15 months) of drinking-water containing arsenic (1:1 arsenite to arsenate) at 3.2 mg/L in male BALB/c mice (5–14 experimental animals, 5–10 control animals). Groups of arsenic-exposed mice and unexposed controls were killed at 3, 6, 9, 12 and 15 months for examination of hepatic histology and certain biochemical parameters of oxidative stress. Statistically significant decrements in body weight were observed in the exposed animals at 12 months and 15 months, without significant differences in the amount of food or water consumption between exposed and control groups. No abnormal hepatic morpho-

logy was observed by light microscopy during the first 9 months of exposure to arsenic, but at 12 months, 11/14 mice in the experimental group exhibited hepatocellular degeneration and focal mononuclear cell collection. After 15 months, exposed mice displayed evidence of hepatocellular necrosis, intralobular mononuclear cell infiltration, Kupffer cell proliferation and portal fibrosis. Hepatic morphology was normal in all control mice. Biochemical changes, consistent with oxidative stress, preceded the overt histological pathology. Hepatic GSH was significantly reduced after 6 months, in a time-related manner; the hepatic activities of enzymes related to GSH homeostasis, namely G6PDH, GST, GSH reductase, GPx and catalase were also reduced in a time-related manner (at 9, 12 and 15 months). There was a progressive, time-dependent increase in lipid peroxidation, as demonstrated by increased production of malondialdehyde, and concomitant time-dependent damage to hepatocellular plasma membranes, as demonstrated by decreases in membrane Na^+/K^+ ATPase activity. Depletion of GSH may result in the accumulation of free radicals that initiate lipid peroxidation and biochemical damage by covalent binding to macromolecules. Biochemical changes observed in this long-term in-vivo animal feeding experiment suggest that the adverse histological effects of arsenic on the liver may be mediated through oxidative stress.

Ishinishi *et al*. (1980) studied the chronic toxicity of arsenic trioxide in rats with special reference to liver damage. Four groups of male adult Wistar rats were given distilled water containing 0, 0.125, 12.5 or 62.5 ppm arsenic trioxide orally for 7 months and were thereafter given distilled water with no arsenic trioxide for 4 months. Despite no difference in growth among the four groups of rats, chronic exposure to arsenic trioxide induced not only liver injury but also dose-dependent proliferation of the bile duct with chronic angitis. The liver injury was characterized by degenerative changes in hepatocytes, such as cloudy swelling, disordered trabeculae or irregularity of hepatocyte tracts, and spotty coagulative necrosis with infiltration of round cells. Sarin *et al*. (1999) demonstrated hepatic fibrosis and fibrogenesis following chronic ingestion of arsenic in Swiss albino mice fed arsenic daily (120, 240, 360 or 500 mg/L). A significant increase in hepatic collagen and its deposition in the extracellular matrix, an expression of hepatic fibrosis, were seen in arsenic-treated mice compared with controls. Hepatic 4-hydroxyproline levels, indicative of fibrogenesis, were increased four- to 14-fold with different doses of arsenic compared with controls.

Effects on levels of GSH and some related enzymes in tissues after acute exposure to arsenic were studied in rats by Maiti and Chatterjee (2001). Male Wistar rats, maintained on either an 18% or 6% protein (casein) diet, received an intraperitoneal injection of sodium arsenite at its LD_{50} dose (15.86 mg/kg bw). One hour after exposure to arsenic, the GSH concentration was significantly depleted and lipid peroxidation was increased in both the high- and low-protein diet groups. Acute exposure to arsenic significantly increased GPx activity in the liver in both groups. GST activity was significantly decreased in the liver of the animals fed 18% protein, whereas it increased in the kidneys of both groups. No significant change in GSH reductase or G6PDH activity in the liver and kidneys was observed. In this study, liver as a whole seemed to be more affected in terms of level of

GSH and GST activity. The animals fed 6% protein appeared to be less affected in terms of tissue arsenic concentration, level of GSH, level of lipid peroxidation and GST activity compared with those fed 18% protein. This might be due to the deficiency in tissues of possible target proteins for arsenic binding and a lesser availability of specific amino acid to synthesize different stress proteins in the animals fed 6% protein.

As reviewed by Del Razo *et al.* (2001b), a variety of genes related to base excision repair and oxidative stress are commensurately up-regulated by nanomolar concentrations of inorganic arsenic. Reactive oxygen species induced by low levels of As^{III} or As^{V} increase the DNA-binding activity of activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) in cultured aortic endothelial cells (Barchowsky *et al*., 1996), human MDA-MB-435 breast cancer and rat H411E hepatoma cells (Kaltreider *et al.*, 1999) and precision-cut lung slices (Wijeweera *et al.*, 2001). This results in stimulation of cell proliferation and up-regulation of gene expression including that of mdm2 protein, which is a key regulator of the critical tumour-suppressor gene *p53* (Germolec *et al.*, 1996; Hamadeh *et al.*, 1999). In contrast, high levels of inorganic arsenic inhibit the activation of NF-κB and cell proliferation and induce apoptosis in human acute myelogenous leukaemia cells, human embryonic kidney (HEK 293) cells and human bronchial epithelial (BEAS 2B) cells (Estrov *et al.*, 1999; Roussel & Barchowsky, 2000). Based on results obtained in NIH 3T3 cells exposed to arsenic, Chen *et al.* (1998) have suggested that apoptosis is triggered by generation of H_2O_2 through the activation of flavoprotein-dependent superoxide-producing enzymes (e.g. NADPH oxidase) and the increase in superoxide levels in cells. The event probably acts as a mediator to induce apoptosis through the release of cytochrome c from the mitochondria to cytosol, the activation of caspase 3 and the degradation of poly(ADP-ribose) polymerase (PARP) leading to DNA fragmentation (Chen *et al.*, 1998).

(iv) *Stress proteins*

Exposure to arsenicals either *in vitro* or *in vivo* in a variety of model systems has been shown to induce a number of the major stress protein families such as heat-shock proteins. Among them are members with a low molecular weight, such as metallothionein and ubiquitin, and others with masses of 27, 32, 60, 70, 90 and 110 kDa. In most cases, the induction of stress proteins depends on the capacity of the arsenic compound to reach the target, its valence and the type of exposure, with arsenite being the strongest inducer of most heat-shock proteins in several organs and systems. Induction of heat-shock proteins is a rapid dose-dependent response (1–8 h) to acute exposure to arsenite. Thus, the stress response appears to be useful for monitoring toxicity resulting from a single exposure to arsenite. The capacity of arsenic compounds to modulate the expression and/or accumulation of stress proteins has been studied in normal and transformed cell lines by Caltabiano *et al*. (1986), Keyse and Tyrrell (1989), van Wijk *et al*. (1993), Wu and Welsh (1996) and Wijeweera *et al*. (2001) and has been reviewed by Bernstam and Nriagu (2000) and Del Razo *et al*. (2001b).

Metallothionein is a low-molecular-weight, cysteine-rich, metal-binding protein that has been propounded to play an important role in the homeostasis of essential metals, in

the detoxication of heavy metals and in the scavenging of free radicals. Moreover, it is a small protein easily induced by heavy metals, hormones, acute stress and a variety of chemicals. Twenty of the 61 amino acid residues in mettalothionein molecules are cysteinyl residues, all of which are involved in metal binding (Sato & Bremner, 1993; National Research Council, 1999).

The induction of metallothionein is observed following oral administration; the doses of organic arsenic compounds (MMA and DMA) required for its induction are one order higher than those of inorganic arsenic compounds (As^{III} and As^{V}). Only a small portion of the arsenic dose was found to be associated with the metallothionein fraction, which there fore does not protect against arsenic toxicity by binding the metal (Maitani *et al*., 1987). Rather, because of its high sulfhydryl content, it has also been suggested that metallothionein reacts with organic free radicals and electrophiles (Klaassen & Cagen, 1981). Indeed, metallothionein can serve as a sacrificial scavenger for superoxide and hydroxyl radicals *in vitro* (Thornalley & Vašák, 1985). It is induced by metal chemicals that produce oxidative stress (Bauman *et al*., 1993) and has been shown to protect against oxidative damage (Sato & Bremner, 1993).

The effect of various arsenic forms on the tissue concentrations of metallothionein was determined in male CF-1 mice (25–30 g) injected subcutaneously with various doses of As^{III} (55–145 μmol/kg bw), As^{V} (165–435 μmol/kg bw), MMA (100–7250 μmol/kg bw) or DMA (2750–10 250 μmol/kg bw) (Kreppel *et al*., 1993). Controls were injected with an equal volume (0.01 mL/g bw) of saline. Metallothionein content in hepatic cytosol was quantified by the cadmium–haemoglobin assay. As^{III} was found to be a potent inducer of hepatic metallothionein, producing a 30-fold increase at a dose of 85 μmol/kg. In comparison, it took three-, 50- and 120-fold higher molar amounts of As^{V}, MMA and DMA, respectively, to produce a similar effect. MMA produced the largest increase in hepatic metallothionein (80-fold), followed by As^{III} (30-fold), As^{V} (25-fold) and DMA (10-fold). However, none of the compounds induced metallothionein in mouse primary hepatocyte cultures, suggesting that arsenicals may be considered as indirect inducers of metallothionein. Both metallothionein-I (MT-I) and metallothionein-II (MT-II) protein isoforms were commensurately induced by As^{III}, As^{V} and MMA. Induction of metallothionein by As^{III} was further characterized following subcutaneous administration of arsenite (85 μmol/kg). Induction of hepatic metallothionein peaked at 24 h. As^{III} also increased metallothionein in kidney, spleen, stomach, intestine, heart and lung and the most marked increase occurred in the liver. MT-I mRNA increased 24-, 52- and 11-fold at 3, 6 and 15 h after administration of As^{III}, respectively. This induction profile is similar to that observed after exposure to zinc or cadmium. This study showed that arsenic compounds are effective inducers of metallothionein *in vivo* and that their potency and efficacy are dependent on the chemical form of arsenic. As^{III} is a potent inducer of hepatic metallothionein for both MT-I and MT-II and this effect is associated with an increase in metallothionein mRNA, suggesting that the mechanism of this induction appears to be due, at least in part, to increased metallothionein gene transcription.

In a recent study, Liu *et al*. (2000) demonstrated that MT-I/II-null mice are more sensitive than wild-type mice to the hepatotoxic and nephrotoxic effects of chronic oral administration or injection of inorganic arsenicals. Groups of 4–6 male and female MT-I/II-null mice and corresponding wild-type mice, aged 6–8 weeks, were provided drinking-water containing As^{III} at concentrations of 7.5, 22.5 or 45 mg/L, or As^{V} at concentrations of 37.5 or 75 mg/L, or were injected subcutaneously in the dorsal thoracic midline with 10 mL/kg bw saline containing As^{III} at doses of 10 and 30 µmol/kg bw or As^{V} at a dose of 100 µmol/kg bw once daily on 5 days per week for 15 weeks. Control mice received tap-water or were injected with the same volume of saline. Chronic exposure to arsenic produced only modest increased tissue concentrations of metallothionein (two- to fivefold) in wild-type but not in MT-null mice, either following repeated injections or following oral administration. Arsenic by both routes produced damage to the liver (fatty infiltration, inflammation and focal necrosis) and kidney (tubular cell vacuolization, inflammatory cell infiltration, glomerular swelling, tubular atrophy and interstitial fibrosis) in both MT-null and wild-type mice. However, in MT-null mice, the pathological lesions were more frequent and severe compared with those in wild-type mice in either liver or kidney. This was confirmed biochemically, in that, at the higher oral doses of As^{V}, the levels of blood urea nitrogen, an indicator of kidney injury, were increased to a greater extent in MT-null mice (60%) than in wild-type mice (30%). However, As^{III} resulted in elevated levels of blood urea nitrogen in MT-null mice only. Chronic exposure to arsenic produced a two- to 10-fold increase in levels of serum interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), with greater increases seen after repeated injections than after oral exposure; again, MT-null mice had higher levels of serum cytokines than wild-type mice, following repeated injections of arsenic, but not after oral exposure. Repeated injections of arsenic also decreased hepatic GSH up to 35% but had no effect on hepatic GPx or GSH reductase activities. MT-null mice were more sensitive than wild-type mice to the effect of GSH depletion by As^{V}. Hepatic caspase 3 activity was increased (two- to threefold) in both wild-type and MT-null mice, indicating apoptotic cell death. The study demonstrated that chronic exposure to inorganic arsenic produced injuries to multiple organs, and that MT-null mice are generally more susceptible than wild-type mice to arsenic-induced toxicity regardless of route of exposure, suggesting that metallothionein could be a cellular factor in protecting against chronic arsenic toxicity.

Kato *et al*. (2000) reported from earlier studies the induction and accumulation of heat-shock protein-72 (Hsp72) in the cell nuclei of human alveolar type II (L-132) cells and DNA damage following exposure to DMA^{V} (Kato *et al.*, 1997). They also found that the accumulation of Hsp72 in cell nuclei was related to the suppression of apoptosis (Kato *et al.*, 1999). Referring to reports indicating that Hsp72 might be involved in the tumorigenic process through the function of apoptosis (Jäättelä, 1999), they assumed that Hsp72 induced by dimethylarsenics may play an important role in DNA damage and tumorigenesis. They therefore investigated whether Hsp72 was induced and accumulated in the lung, a target organ for tumorigenesis, following administration of DMA^{V} to mice. Five-week-old male A/J mice were injected intraperitoneally with DMA^{V} (100–600 mg/kg) or

arsenite (5 mg/kg bw) and then killed. Lung, kidney, liver and spleen were excised, homogenized and immunoblotting analysis was performed with anti-Hsp72 monoclonal antibody. Hsp72 in lung was also investigated immunohistochemically. Forty-eight hours after exposure to DMA, Hsp72 was observed in the lung and in the kidney, but not in the liver or spleen. Hsp72 was also detected by immunohistochemical analysis in the nuclei of alveolar flat cells containing capillary endothelium, in the lungs of DMA-treated mice. This result may be consistent with those observed in previous studies showing that oral administration of DMA to mice induces a preferential increase in heterochromatin in the vesicular endothelium of the lung, an early morphological change in the development of pulmonary carcinomas (Nakano *et al.*, 1992; Hayashi *et al.*, 1998). Kato *et al.* (2000) suggested that the increase and accumulation of Hsp72 following administration of DMA to mice occur specifically in target organs for the carcinogenesis of arsenic. It appears that arsenic compounds regulate the expression of the major families of heat-shock proteins and that inorganic As^{III} is the most potent inducer of these proteins (Del Razo *et al.*, 2001b).

Stress-related gene expression in mice treated with inorganic arsenic has been studied by Liu, J. *et al*. (2001). Adult male 129/Sv mice, aged 6–8 weeks, were injected subcutaneously in the dorsal thoracic midline with 100 μmol/kg bw As^{III}, 300 μmol/kg bw As^{V} or the same volume of saline (10 mL/kg bw). To examine stress-related gene expression, livers were removed 3 h after injection of arsenic to extract RNA and protein. The Atlas Mouse Stress/Toxicology array revealed that the expression of genes related to stress — DNA damage and repair-responsive genes — and metabolism were altered by acute exposure to arsenic. Expression of H-O-1, a hallmark for arsenic-induced stress, was increased 10-fold, together with increases in heat shock protein-60 (Hsp60), the DNA damage-inducible protein GADD45 and the DNA excision repair protein ERCC1 and growth arrest. Down-regulation of certain cytochrome P450 drug-metabolizing enzymes occurred after treatment with arsenic. Because the AP-1 complex is associated with stress-related gene activation, the effect of arsenic on AP-1 complex activation was examined. A multiprobe RNAse protection assay revealed the activation of the c-Jun–AP-1 transcription complex after treatment with arsenic. Western blot analysis further confirmed the enhanced production of arsenic-induced stress proteins such as H-O-1, Hsp70, Hsp90, metallothionein, metal-responsive transcription factor, NF-κB and c-Jun–AP-1. Increases in caspase 1 and cytokines such as TNF-α and macrophage inflammatory protein-2 were also evident. Activation of caspase has been propounded to play a role in arsenic-induced apoptosis (Chen *et al.*, 1998), and induction of inflammatory cytokines is another important aspect of arsenic toxicity. The results of this study profiled gene expression patterns in mice treated with inorganic arsenicals. The altered gene expressions following acute exposure to arsenic *in vivo* include stress-related components —DNA damage and repair-responsive genes — activation of transcription factors such as the AP-1 complex and an increase in proinflammatory cytokines.

Expression of shock proteins is regulated by a complex mechanism that requires the integration of multiple signal pathways. The inter-relationships among stress signalling,

cell death and oncogenesis after exposure to arsenic need further research (Del Razo *et al*., 2001b).

(v) *Immunotoxicity*

Although many studies have evaluated the immunological effects of environmental toxic substances such as lead, cadmium and mercury, only a few studies on arsenic have been reported.

Yoshida *et al*. (1986) reported immunological effects of arsenic compounds on mouse spleen cells *in vitro*. Spleens from male C57BL/6N mice were removed aseptically, and sterile viable spleen cells were cultured with 20 µL/mL of an arsenic solution (at concentrations of 1–500 ng/mL, 0.01–50 µg/mL and 0.1–500 µg/mL, for sodium arsenite, sodium arsenate and DMA, respectively). Saline (0.9% NaCl) was added to control cultures. For plaque-forming cell (PFC) response, spleen cells ($2.5–3.0 \times 10^5$ cells/mL) were cultured in triplicate and incubated with 8×10^6 sheep erythrocytes, and the number of direct (immunoglobulin M) PFCs were enumerated. Spleen cells (2.5×10^6 cells/mL) were also cultured for 48 h with or without the mitogens, phytohaemagglutinin P (PHA) or lipopolysaccharide ω. At high doses, the three arsenic compounds (sodium arsenite, sodium arsenate and DMA) suppressed the PFC response to sheep erythrocytes and the proliferative response to mitogens whereas, at low doses, they enhanced both responses. In other studies, the authors have demonstrated that this immunoenhancing effect of arsenic on PFC response to sheep erythrocyte is not attributable to the augmentation of lymphocyte function, but to the cytotoxicity of arsenic against precursors of suppressor T cells (Yoshida *et al.*, 1987). The concentration at which each arsenic compound exerted the modulatory effects on both responses differed, and was correlated to the general toxicity of each compound.

A pilot study on arsenic-exposed humans was carried out by Ostrosky-Wegman *et al*. (1991) to determine the lymphocyte proliferation of kinetics and genotoxic effects. The exposed group comprised 11 individuals (nine women and two men) from Santa Ana, State of Coahuila, Mexico, where the drinking-water contained 0.39 mg/L arsenic (98% in pentavalent form and the rest in trivalent form). The non-exposed group (13 individuals; 11 women and two men) was chosen from Nuevo Leon, State of Coahuila, where levels of arsenic in the drinking-water ranged from 0.019 to 0.026 mg/L during 1987–89; while sampling was performed, the levels rose to 0.060 mg/L because of new piping that linked several towns in the area. Venous blood samples were taken and lymphocyte cultures were rapidly processed. The analysis of chromosomal aberrations and sister chromatid exchange was performed in 100 consecutive first-division metaphases and in 30 consecutive second-division metaphases, respectively, all with 46 centromeres. The proportion of first, second, third and subsequent metaphases was determined in 100 consecutive mitoses to study the kinetics of proliferation. The highly exposed group excreted greater amounts of arsenic in urine; neverthelesss, the *Bacillus subtilis* rec-assay for genetic damage induced by urine samples showed negative results. There was a significant difference in cell-cycle kinetics between the groups: the average generation time

was longer in the highly exposed group. The lag in lymphocyte proliferation could mean an impairment of the cellular immune response due to exposure to arsenic.

Because inhibition of lymphocyte proliferation has been used to identify agents that depress the cellular immune response, Gonsebatt *et al*. (1992) investigated *in vitro* the effect of arsenic on human lymphocyte stimulation and proliferation using concentrations of arsenic similar to those found in blood. When human lymphocytes collected from healthy donors (two men, two women) were exposed to arsenite and arsenate (10^{-7}, 10^{-8} and 10^{-9} M) during culture and harvested after 24 h, a dose-related inhibition of proliferation was observed. Cultures were also treated with 10^{-7} M arsenite and arsenate for 2, 6 and 24 h at the beginning of culture in the presence or absence of PHA. Inhibition of PHA stimulation and proliferation was directly related to the length of treatment with arsenic. The results show that, at the concentrations tested, arsenite and arsenate impaired lymphocyte stimulation and proliferation and confirm that chronic exposure to arsenic can affect the proliferation of whole-blood lymphocytes.

A human monitoring study was subsequently carried out by Gonsebatt *et al*. (1994) to explore the effect on lymphocyte proliferation of chronic exposure to arsenic via drinking-water. Blood and urine samples were taken from 33 volunteers from a town where levels of arsenic in the drinking-water averaged 412 µg/L and from 30 subjects from a matched group with similar socioeconomic status, who drank water with an average level of 37.2 µg/L arsenic. Exposure was assessed by questionnaire and by determining the levels of arsenic in urine and water samples. Peripheral blood lymphocyte proliferation was evaluated at different culture times using labelling (radioactive thymidine incorporation), mitotic and replication indexes as end-points. No significant differences were seen for either labelling or mitotic indices, except for mitotic index in 72-h cultures (higher in the exposed group) and for labelling index (lower) in men and women with skin lesions versus those without lesions. Significant decreases in replication index were seen for exposed women but not for men. Correlations between labelling and mitotic indices showed that progression from the initial S- to M-phase is altered in exposed individuals. The results obtained corroborate the slower cell kinetics found previously in the pilot study by Ostrosky-Wegman *et al*. (1991).

From the preceding reports, it appears that inorganic arsenic is immunotoxic, but the mechanism of immune suppression is not clear. Harrison and McCoy (2001) showed that arsenite inhibits the enzymatic activity of lysosomal protease cathepsin L (CathL) in cultures of the murine antigen-presenting B-cell line TA3 and in lysates from unexposed TA3 cells *in vitro*. Arsenite also significantly inhibits purified CathL. This enzyme plays an important role in antigen processing, the mechanism by which antigen-presenting cells cleave foreign protein antigens to peptides to stimulate a T-cell response. Deficient proteolysis may lead to diminished immune responses. Arsenite suppressed enzymatic activity within TA3 cells after 4 h of exposure without affecting cell viability. Kinetic analyses revealed that arsenite was a reversible, partially noncompetitive inhibitor of CathL with a Ki of 90 µM for TA3-derived and 120 µM for the purified enzyme. Indeed, upon addition of excess dithiotheitol, the enzyme activity of CathL was restored; the value

of Ki was comparable to that of the arsenite concentration that maximally decreased CathL in viable TA3 cells after 4 h of exposure. However, an 18-h exposure to arsenite triggered massive cell death at concentrations that were substantially lower than those required for enzymatic inhibition. Morphological analysis (chromatin condensation, cell shrinkage) and annexin V staining showed that arsenite-exposed TA3 cells underwent apoptosis within 18 h and early stages of apoptosis began within 4 h, indicating that arsenic causes apoptosis independent of CathL. Although whether in-vivo exposure to arsenic causes apoptosis in lymphoid organs has not been assessed, these findings suggest that apoptosis could be a major mechanism of arsenic-induced immunosuppression.

4.3 Reproductive and developmental effects

4.3.1 *Humans*

In a case–control study, Zierler *et al.* (1988) compared 270 cases of infants born with congenital heart disease and 665 controls from Massachusetts (USA). The proportional odds ratio, adjusted for all measured contaminants, source of water and maternal education, was not elevated for any congenital heart disease in relation to exposure to arsenic above the detection limit of 0.8 µg/L. However, for a specific malformation, coarctation of the aorta, there was a significant proportional odds ratio of 3.4 (95% CI, 1.3–8.9). The exposure was low, the 90th percentile level being 1 µg/L.

In a case–control study, Aschengrau *et al.* (1989) examined 286 women who experienced spontaneous abortions and 1391 controls from Boston, MA (USA), in relation to the content of their water supplies. An adjusted odds ratio of 1.5 was found for the group with the highest arsenic concentrations. [However, this exposure group had low levels of arsenic in water (1.4–1.9 µg/L), close to or lower than laboratory analytical detection limits, and the possibility of chance or unaccounted confounders could not be discounted.]

An ecological study in an area of south-east Hungary with exposure to arsenic from drinking-water examined the rates of spontaneous abortions and stillbirths for the period 1980–87. Two populations were compared: one from an area with levels of arsenic in drinking-water > 100 µg/L (n = 25 648 people) and one control area with low levels of arsenic (n = 20 836). [No information on analytical method, timing or frequency of sampling was available.] The incidences of both outcomes were significantly higher in the exposed groups, with a 1.4-fold increase in spontaneous abortions (p = 0.007) and a 2.8-fold increase in stillbirths (p = 0.028) (Borzsonyi *et al.*, 1992). [Although both populations were stated to have several similar characteristics, such as smoking, lifestyle, occupation and socioeconomic status, no data were provided, and other important factors such as maternal age were not considered. Furthermore, no mention was made of other potential environmental exposures.]

An ecological study conducted in the USA investigated mortality from vascular diseases in the 30 counties with the highest average levels of arsenic in drinking-water for

the period 1968–84. The arsenic levels ranged up to 92 μg/L in Churchill County, NV. SMRs were based on comparison with the population of the USA. When counties were grouped into three arsenic-exposure categories, defined as 5–10, 10–20 and > 20 μg/L, there appeared to be an increase in mortality from congenital anomalies of the heart only for females in the highest exposure group (SMR, 1.3; 95% CI, 1.0–1.8) and for both sexes for congenital anomalies of the circulatory system (female SMR, 2.0; 95% CI, 1.0–3.4; male SMR, 1.3; 95% CI, 0.7–2.4) (Engel & Smith, 1994).

A retrospective ecological study examined infant mortality rates in three Chilean cities over a 46-year period (1950–96). Antofagasta, in northern Chile, experienced very high levels of arsenic in drinking-water for a period of 12 years. In 1958, a new water source, which contained arsenic concentrations of around 800 μg/L, was introduced as the main supply of public water. In 1970, because of the overt signs of arsenicism observed in several studies, a plant for the removal of arsenic was installed, and levels decreased initially to around 110 μg/L, and then gradually over time to around 40 μg/L (see Table 18). The changes in late fetal, neonatal and post-neonatal mortality rates over time in Antofagasta were compared with those in Valparaiso, another Chilean city with similar demographic characteristics but with low levels of arsenic. A temporal relationship was observed between the period of high arsenic contamination and a rise in neonatal mortality rates, in particular in Antofagasta, whereas the other city had a fairly steady decline in infant mortality (Hopenhayn-Rich *et al.*, 2000). [Data on other contaminants or factors related to infant mortality were not presented, but the temporal relationship suggests a role for exposure to arsenic.]

A retrospective survey in Bangladesh compared several outcomes in women exposed to high (mean, 240 μg/L; $n = 96$) and low (< 20 μg/L; $n = 96$) concentrations of arsenic in drinking-water. Rates of spontaneous abortions, stillbirths and pre-term births were 2.9 ($p = 0.08$), 2.24 ($p = 0.046$) and 2.54 ($p = 0.018$) times higher, respectively, in the high-exposure group than in the low-exposure group. The groups were comparable in terms of age, socioeconomic status, level of education and age at marriage (Ahmad *et al.*, 2001). [This study was based on recall of previous pregnancies, however, and ascertainment of the outcomes was not clearly defined.]

4.3.2 *Experimental systems*

(*a*) *Developmental toxicity*

(i) In vivo

Inorganic arsenic is toxic to mouse and hamster embryos and fetuses after oral or intraperitoneal administration to the dams, with arsenite being three- to 10-fold more potent than arsenate. The embryos and fetuses of hamsters are more sensitive to this effect than those of mice. The toxicity is characterized by decreases in fetal weight, crown–rump length, embryo protein content and the number of somites and by growth retardation and lethality (Baxley *et al.*, 1981; Hood & Harrison, 1982; Hood & Vedel-Macrander, 1984; Carpenter, 1987; Domingo *et al.*, 1991; Wlodarczyk *et al.*, 1996).

Sodium arsenite was given by gavage to CD-1 mice on one of days 8–15 of gestation at doses of 20, 40 or 45 mg/kg bw. The lowest dose had no effect. The two highest doses produced 19 and 36% incidences of maternal deaths, respectively, and also decreased fetal weight and increased the incidence of resorptions. Arsenite-induced lethality was dependent on dose and day of gestation (Baxley *et al*., 1981).

In hamsters, sodium arsenite administered orally (20–25 mg/kg) caused less fetal mortality than parenteral dosing (2.5–5 mg/kg) (Hood & Harrison, 1982).

Nemec *et al.* (1998) evaluated the developmental toxicity of arsenate administered by oral gavage to CD-1 mice and New Zealand white rabbits. Rabbits received doses of 0, 0.19, 0.75 or 3.0 mg/kg bw per day on gestation days 6–18 and mice received 0, 7.5, 24 or 48 mg/kg per day on gestation days 6–15. Increased fetal resorptions and decreased fetal weight were observed only at exposure levels resulting in maternal toxicity (severely decreased weight gain, mortality).

A single intravenous administration of MMA^V (disodium salt) or DMA^V (sodium salt) on day 8 of gestation at dose levels of 20–100 mg/kg elicited a low resorption rate (≤ 10%) in pregnant hamsters (Willhite, 1981). Higher doses of DMA (sodium salt, 900–1000 mg/kg) administered intraperitoneally to pregnant hamsters on one of days 8–12 of gestation induced higher resorption rates, ranging from 30–100% of the litters. MMA^V (500 mg/kg) was less toxic than DMA after intraperitoneal administration, with 6–21% of the litters resorbed. Fetal growth was retarded after administration of MMA on days 9, 10 or 12 of gestation (Hood *et al.*, 1982).

DMA^V administered orally to pregnant mice (200–600 mg/kg per day) and rats (7.5–60 mg/kg per day) on days 7–16 of gestation resulted in significant fetal mortality in mice at 600 mg/kg per day and rats at 50–60 mg/kg per day. A significant decrease in fetal weight gain was observed in mice at 400–600 mg/kg and rats at 40–60 mg/kg (Rogers *et al*., 1981).

Inorganic arsenic elicits teratogenic effects in mice (Hood & Bishop, 1972; Baxley *et al.*, 1981; Morrissey & Mottet, 1983; Wlodarczyk *et al.*, 1996), rats (Fisher, 1982) and hamsters (Hood & Harrison, 1982; Carpenter, 1987) at levels of tens of milligrams per kilogram body weight after oral or intraperitoneal administration. In these studies, the major teratogenic effect induced is cephalic axial dysraphic disorder or neural tube defect. The defect is characterized by exencephaly and encephalocele, which are characterized by non-closure and partial closure of the cephalic neural folds, respectively. Other malformations that occur to a minor extent include fused ribs, renal agenesis, micromelia, facial malformations, twisted hindlimb, microphthalmia and anophthalmia. The malformations are dose- and gestational age-dependent. Sodium arsenite is more potent than sodium arsenate in inducing a teratogenic response, and intraperitoneal administration of arsenic is more effective than oral administration.

Histological studies of the developing urogenital system in rat embryos after intraperitoneal administration of arsenate to pregnant rats revealed that the first observable change is a retardation in the growth of the mesonephric duct. This retardation led to the absence of the ureteric bud (which arises from the mesonephric duct) and resulted in the

absence of the vas deferens, seminal vesicle and part of the epididymis (Burk & Beaudoin, 1977).

Administration of inorganic arsenic to mice on days 7–9 of gestation results in neural tube defects in the developing organism. The time most sensitive to arsenate in mouse embryos is when the dams are administered the chemical on day 8. Of the fetuses that survived a single dose of sodium arsenate (45 mg/kg) administered intraperitoneally to dams on day 8, 65% or more were exencephalic. After administration of a similar dose of arsenate on day 7 or 9, 3% or less of the surviving fetuses were exencephalic (Morrissey & Mottet, 1983).

The neural tube defects seem to result from an apparent arsenic-induced arrest or delay in neural-fold apposition. Takeuchi (1979) examined the changes induced by an embryo-lethal dose of arsenate (30 mg/kg) administered to pregnant rats intraperitoneally on day 9 of gestation. At 4 h after exposure, some cellular necrosis was seen in the neuroectoderm and mesoderm of the embryos. By 12 h, abnormal mitotic and interphase cells were observed in both tissues, and necrotic cells and debris from these cells were also present. By 24 h, neurulation had stopped, as evidenced by the presence of the V-shaped neural fold that is normally closed by this time.

In studies by Morrissey and Mottet (1983), pregnant mice were killed 6–21 h after intraperitoneal administration of sodium arsenate (45 mg/kg) on day 8 of gestation. Neural folds were widely separated and not positioned for closure in the prospective hindbrain. Necrotic debris was also found primarily in the neuroepithelium of the prospective forebrain and sometimes in the mesenchyme, but it was not clear if this was the main lesion associated with exencephaly.

Fisher (1982) examined the effect on the development of embryos of sodium arsenate (45 mg/kg bw) administered intraperitonally to pregnant rats on day 10 of gestation. These rats were killed 4 h or 24 h after injection. The embryos were removed and the macromolecule levels were determined immediately, or at 24 h or 42 h after being placed in culture media. In-utero exposure to arsenate for 4 h did not affect the macromolecule levels. A 24-h in-utero exposure to arsenate resulted in a significant decrease in DNA, RNA and protein accumulation at the beginning of cultivation and after 24 h in culture. However, after 42 h in culture, protein levels had recovered. After 24 h in culture, morphological changes in the 24-h exposed embryos included a failure to rotate to a ventroflexed position, failure of closure of the anterior neuropore, no establishment of visceral yolk sac circulation, and no fusion of the allantoic sac in placental formation. The latter effect may reflect problems in the formation of the urogenital system.

Nemec *et al.* (1998) observed no teratogenic effects in mice or rabbits receiving daily oral administrations of 0–48 or 0–3 mg/kg bw arsenate on gestation days 6–15 or 6–18, respectively.

MMA^V (disodium salt, 20–100 mg/kg) and DMA^V (sodium salt, 20–100 mg/kg) induced a low percentage of fetal malformations (≤ 6%) after intravenous administration on day 8 of gestation to pregnant hamsters. The effects were characterized by fused ribs,

renal agenesis or encephalocele, with the latter anomaly was observed only with DMA. Neither MMA nor DMA caused maternal toxicity (Willhite, 1981).

The effect of continuous oral exposure of pregnant mice (200, 400, 600 mg/kg per day) and rats (7.5–60 mg/kg per day) to DMA^V during days 7–16 of gestation was examined by Rogers *et al*. (1981). In mouse fetuses, cleft palate was the major teratogenic response to DMA and was observed at the two highest doses. There was also a significant decrease in the incidence of supernumerary ribs. In the mid-dose group, four mouse fetuses had irregular palatine rugae. In rats, the average number of sternal and caudal ossifications was decreased at the two highest doses and the percentage of irregular palatine rugae increased significantly with dose. An increase in fetal lethality occurred at the highest dose in mice (39.8%) and at the two highest doses in rats (32.9 and 65.4%).

(ii) In vitro

Muller *et al.* (1986) examined the effect of sodium arsenite on mouse embryos at the two-cell pre-implantation stage, which is approximately 30–32 h after conception. Arsenite-induced lethality occured at a concentration of 100 µmol/L. After implantation, arsenite and arsenate are toxic (decreases in crown–rump length, number of somites, protein content, head length, yolk sac diameter) and lethal to embryos of mice (Chaineau *et al.*, 1990; Tabacova *et al.*, 1996) and rats (Mirkes & Cornel, 1992; Mirkes *et al.*, 1994). Tabacova *et al.* (1996) observed that as gestational age at which the mouse embryos were isolated and exposed to arsenic increased, so did resistance to toxicity or lethality. As in the in-vivo studies, arsenite was more potent than arsenate.

Inorganic arsenic is teratogenic to cultured mouse embryos (day 8), with sodium arsenite (1–4 µmol/L) being approximately 10-fold more effective than sodium arsenate (10–40 µmol/L) after a 48-h incubation. The most sensitive in-vitro effect of arsenic is hypoplasia of the prosencephalon. Other effects include failure of neural tube closure and development of limb buds and sensory placode, somite abnormalities and, in arsenate-exposed embryos, hydropericardium (Chaineau *et al.*, 1990).

Arsenite inhibits chondrogenesis in chick limb bud mesenchymal cells, with complete inhibition at 25 µmol/L. Arsenate was ineffective at concentrations up to 200 µmol/L but, when added with arsenite, gave an apparent dose-dependent additive effect (Lindgren *et al.*, 1984).

Sodium arsenite (50 µmol/L) induces dysmorphology in rat embryos (10 days old) after a 2.5-h exposure followed by a 21.5-h incubation period without arsenic. This effect is characterized by hypoplastic prosencephalon, mild swelling of the rhombencephalon and abnormal somites and flexion of the tail (Mirkes & Cornel, 1992; Mirkes *et al.*, 1994).

Tabacova *et al.* (1996) examined the teratogenicity of arsenite (1–30 µmol/L) and arsenate (5–100 µmol/L) in mouse embryos isolated from pregnant dams on day 9 of gestation. The embryos were incubated with various concentrations of arsenic, for different lengths of time and at various stages of somite development. Treatment with arsenic led to non-closure of the neural tube, collapsed neural folds, prosencephalic hypoplasia, anophthalmia, pharyngeal arch defects and abnormal somites. The malformation rates were

dependent on the dose and oxidation state of arsenic. Arsenite was generally three to four times more potent than arsenate in inducing these effects. As the age of the embryos advanced, a higher dose of arsenic was required to elicit the effect. The developmental effects most sensitive to inorganic arsenic were forebrain growth, neural tube closure, eye differentiation, axial rotation (dorso- to ventroflexion) and pharyngeal arch development, which were induced by a 1-h exposure to inorganic arsenic.

(*b*) *Gene expression*

Wlodarczyk *et al.* (1996) examined the expression of several transcription factors from embryos isolated from pregnant mice administered sodium arsenate intraperitoneally at 30–45 mg/kg, an approximately lethal dose. Expression of several genes was altered by arsenate administered on day 9 of gestation. This day corresponds to the progression of neural tube closure, which is delayed in embryos exposed to inorganic arsenic. In the neuroepithelium of arsenate-exposed embryos, there was significant down-regulation of *Hox 3.1* and up-regulation of *Pax3*, *Emx-1* and *creb*. Both *Hox 3.1* and *Pax3* play a role in the regulation of neural cellular adhesion molecules, a glycoprotein that affects neural crest cell migration and ultimately neural tube closure (Rutishauser *et al.*, 1988).

(*c*) *Induction of heat-shock proteins*

Arsenic induces the biosynthesis in embryos of several heat-shock proteins that protect cells from its detrimental effects. However, its has been proposed that induction of a heat-shock protein response could alter the normal gene programme for organogenesis (German, 1984).

(i) In vivo

Pregnant mice were administered sodium arsenite (0.5 mg/mouse, approximately 17 mg/kg bw) intraperitoneally on days 9–11 of gestation. Two proteins that were induced were isolated from the embryos and had molecular weights between 45 and 66.2 kDa. Heat-shock treatment of pregnant mice induced one embryonic protein with a molecular weight between 45 and 66.2 kDa and a second with a molecular weight between 66.2 and 92.5 kDa (German *et al.*, 1986). In mice administered sodium arsenite (19 mg/kg) intraperitoneally on day 8 and killed 1 day later, the levels of two proteins, Hsp70 and Hsp105, which are produced constitutively, were increased throughout the embryo. There was a high concentration of these proteins in the neuroepithelial tissue of the embryos after treatment with heat shock or arsenite (Honda *et al.*, 1992).

(ii) *Animal embryos* in vitro

Four proteins with molecular weights of 27, 35, 73 and 89 kDa and their mRNA were induced in chick embryo cells by sodium arsenite (50 µmol/L) or heat shock in a dose- and time-dependent manner. For example, the 35-kDa protein was induced at a concentration of 5 µmol/L sodium arsenite, but the 73- and 89-kDa proteins were minimal at this

concentration. Only the 27-kDa protein was still induced 24–48 h after treatment (Johnston *et al.*, 1980). In chick embryo fibroblasts (10–12 days old), arsenite induced the synthesis of Hsp70A and 70B (Wang & Lazarides, 1984).

Mouse embryo cells (gestation day 11) were exposed to either sodium arsenite (50 μmol/L) for 3 h or heat shock for 10 min, and proteins from cell extracts were analysed by two-dimensional gel electrophoresis. The synthesis of Hsp73 and Hsp105 was increased by both exposures (Honda *et al*., 1992).

In rat embryos (gestation day 10), exposure for 2.5–5 h to an embryotoxic level of sodium arsenite (50 μmol/L) resulted in the induction of three heat-shock proteins (Mirkes & Cornel, 1992). A monoclonal antibody specific for Hsp72 recognized one of the proteins induced by arsenite. Levels of mRNA for these heat-shock proteins were also increased in the embryos after exposure to arsenite. Hsp72 was detected 10 h after exposure, and maximal levels were observed at 24 h. However, Hsp72 was not detected at 48 h, which indicates that this protein is turned over (Mirkes *et al.*, 1994).

(iii) *Human fetal tissue* in vitro

German *et al.* (1986) treated human fetal tissue (gestational age, 77–84 days) with either sodium arsenite (50 μmol/L) for 2 h or heat shock for 6 min. The cells were then examined for induction of heat-shock proteins. Several proteins were induced by both treatments, and two with molecular weights < 45 kDa were induced only by exposure to arsenite.

Honda *et al.* (1992) treated human chorionic villus cells (gestational age, 70–119 days) with sodium arsenite (50 μmol/L) for 3 h or with heat shock for 10 min. In unstressed tissue, Hsp70, Hsp73, Hsp85 and Hsp105 were synthesized constitutively, but their levels were increased after exposure to sodium arsenite or heat.

4.4 Genetic and related effects

The genetic effects of arsenic compounds have recently been reviewed extensively (National Research Council, 1999; Basu *et al*., 2001; Gebel, 2001; National Research Council, 2001; WHO, 2001). In this section, the genotoxicity of arsenic in humans and in experimental animals is dealt with comprehensively. Relevant studies on single and combined mammalian genotoxicity have been included. Data on fungi, plants and *Drosophila* have not been reviewed.

4.4.1 *Humans*

Several studies have investigated the genotoxic effects of arsenic after long-term ingestion via drinking-water, but few studies of occupational exposure to arsenic are available. Exposures were mainly to inorganic arsenic, but since arsenic is methylated in humans, mixed internal exposures to inorganic arsenic and methylated arsenic metabolites predominate. Although MMA and DMA (as sodium salts) have been used in pesti-

cides, this use is currently decreasing and no study was available on the monitoring of human biological effects after occupational exposure to these compounds.

In a pilot study in Mexico, nine women and two men exposed to well-water containing high levels of arsenic (390 μg/L, presumably > 10 years) did not show a significantly higher frequency of chromosomal aberrations or sister chromatid exchange than controls exposed to lower levels of arsenic (11 women and two men; 19–60 μg/L arsenic in well-water). The age range for both groups was 21–62 years. Mutant frequencies at the *HPRT* locus were elevated but not significantly in the high-exposure group (Ostrosky-Wegman *et al*., 1991). In a more recent study, 35 Mexican individuals exposed to well-water containing 408 μg/L arsenic (presumably > 10 years) were compared with 34 controls (well-water concentration, 29.9 μg/L arsenic). The mean age of the two groups was 40.6 years (exposed) and 39.0 years (control), and sex distribution was said to be similar [exact data not supplied]. In the high-exposure group, chromosomal aberrations were significantly elevated, with 0.08 (exposed) versus 0.03 (control) chromosomal aberrations per cell. Moreover, the frequency of micronuclei in buccal and urothelial cells was significantly elevated (average/1000 cells, 2.21 versus 0.56 and 2.22 versus 0.48, respectively) (Gonsebatt *et al*., 1997). Among the exposed individuals, men showed more chromosomal aberrations and higher frequency of micronuclei than women. This difference could be attributed to the fact that men drank more water; in this study country, men work in the fields and, because of the dry climate, drink more water than women. The proportion of smokers was similar in the two groups: 29% of the exposed and 33% of the controls; smoking was not significantly associated with a higher incidence of chromosomal aberrations or micronuclei. People occupationally exposed to putative genotoxins or those who underwent medical treatment were excluded from the study.

No differences in sister chromatid exchange (98 exposed subjects versus 83 controls) or chromosomal aberration (104 exposed versus 86 controls) frequencies were found in the peripheral lymphocytes of subjects exposed to moderate quantities of arsenic in the drinking-water in Nevada (USA). Drinking-water with mean concentrations of 109 μg/L arsenic had been consumed for at least 5 years; control subjects had drunk water containing 12 μg/L arsenic (Vig *et al*., 1984). In the statistical evaluation, sex, age, smoking and putative occupational exposures were controlled for. The population studied was exposed to much lower levels of arsenic than the current study population and arsenic has not been shown to be associated with cancer in blood-forming tissue.

In a more recent study in Nevada (USA), 18 people (mean exposure from drinking-water, 1312 μg/L arsenic > 1 year) showed elevated frequencies of micronuclei in exfoliated bladder cells (2.79/1000 cells) in comparison with 18 control subjects exposed to low levels of arsenic (exposure from drinking-water, 16 μg/L arsenic; 1.57/1000 cells) matched for age, sex and smoking status (Warner *et al*., 1994). Occupation was included as a confounding variable. In contrast, there was no increase in micronucleated buccal cells associated with such high levels of arsenic.

The frequencies of chromosomal aberrations were determined in the peripheral lymphocytes of 32 Finnish subjects (age, 15–83 years; mean, 52 years) after long-term ingestion

of drinking-water containing a median concentration of 410 µg/L arsenic (Mäki-Paakkanen *et al*., 1998) and were compared with those of eight controls (age, 37–76 years; mean, 50 years) from the same village who consumed drinking-water containing < 1 µg/L arsenic. Estimated cumulative median doses of arsenic were 455 and 7 mg per lifetime, respectively. Smoking habits, sex, seafood consumption and residential history were included as confounders in the evaluation. The crude study results did not show elevated frequencies of chromosomal aberrations in arsenic-exposed subjects (6.9 in exposed versus 8.6 in controls) or smokers (6.0 in ex- and current smokers versus 6.9 in never-smokers). However, in the crude and adjusted linear regression analyses, numbers of chromosomal aberrations were significantly associated with levels of arsenic in urine of current users ($r^2 = 0.25$; $p = 0.08$ and $r^2 = 0.27$; $p = 0.04$, respectively).

In a pilot study in Inner Mongolia, 19 residents exposed to arsenic via drinking-water (527.5 µg/L) for 17 years (group average) were compared with 13 control subjects exposed to a low concentration of 4.4 µg/L arsenic (Tian *et al*., 2001). Data on smoking habits, occupation, diet, demographic factors, age and medical status were collected. Frequencies of micronuclei were significantly (3.4-fold) higher in cells from the buccal mucosa and sputum collected from airway epithelium. The increase observed for bladder cells was smaller: 2.7-fold over control for all subjects and 2.4-fold over control for nonsmokers. When smokers were excluded from high-exposure and control groups, the effects of arsenic were greater, although only in buccal and sputum cells, in which sixfold increases in micronuclei frequency occurred.

A nested case–control study was performed in an area endemic for Blackfoot disease in Taiwan, China (Liou *et al*., 1999). A cohort of 686 residents was assembled and, after 4 years, 31 people had developed cancer. Twenty-two blood samples obtained from these subjects at the beginning of the cohort study were successfully processed. A control comparison group was selected from among members of the cohort who had not developed cancer, matched on sex, age, history of residence (residential village) or of drinking artesian well-water and smoking. No differences were found in overall frequencies of sister chromatid exchange. The frequency of chromosomal aberrations was significantly higher among cases, which was due to the induction of chromosome-type but not chromatid-type aberrations. [The Working Group noted that there was no difference in exposure to arsenic (mean duration of drinking artesian well-water) among cases and controls.]

A study in West Bengal, India, compared 45 subjects with cutaneous signs of arsenicism (368 µg/L arsenic in drinking-water) with 21 healthy individuals considered as controls residing in two unaffected districts (5.50 µg/L arsenic in drinking-water) (Basu *et al*., 2002). The frequency of micronuclei was significantly higher in the oral mucosal cells (5.15 versus 0.77 per 1000 cells), urothelial cells (5.74 versus 0.56 per 1000 cells) and peripheral lymphocytes (6.40 versus 0.53 per 1000 cells) of exposed subjects compared with control subjects. The age distribution and socioeconomic status was reported to be similar in the two groups. Exposure of exposed subjects to arsenic via drinking-water had probably been for a mean of 11 years.

In another study, the mean frequency of sister chromatid exchange/cell in human peripheral lymphocytes was not found to be affected by voluntary ingestion of 0.15 g potassium arsenite or poisoning from 1, 10 or 20 g arsenic trioxide. At 20 g arsenic trioxide, the mean frequency of sister chromatid exchange was significantly elevated (Hantson *et al*., 1996). Doses of 10 and 20 g arsenic trioxide significantly increased the number of cells with a high sister chromatid exchange frequency and produced a shift in the distribution of the cells according to frequency of sister chromatid exchange.

Few studies have dealt with the induction of genetic damage in workers exposed to arsenic. Moreover, these subjects were exposed to other genotoxic agents. In the peripheral lymphocytes of nine smelter workers exposed to arsenic and other compounds, a significant increase in chromosomal damage was found, with 87 aberrations per 819 mitoses compared with 13 per 1012 in controls (Beckman *et al*., 1977). In this preliminary report, no data on duration of exposure or age of the workers were given. In a further study, 33 male copper smelter workers (aged 20–62 years) exposed to arsenic and other toxic compounds were studied to determine chromosomal aberrations in peripheral lymphocytes (Nordenson & Beckman, 1982). Internal exposures to arsenic were analysed in urine, but the analytical method was not given. The frequencies of chromosomal aberrations were not associated with age, smoking or degree of exposure to arsenic. Significantly increased frequencies of chromosomal aberrations were found in comparison with 15 male employees (aged 26–60 years) without known occupational exposure to arsenic or other toxic agents: 5.4 aberrations versus 2.1 per 100 cells for gaps and 1.4 aberrations versus 0.1 per 100 cells for chromosome breaks ($p < 0.001$). Chromatid breaks showed a lower significance level (1.3 versus 0.6 per 100 cells [$p < 0.05$]).

Some studies investigated whether arsenic-mediated chromosomal damage *in vivo* is caused by an aneugenic or clastogenic effect (Dulout *et al*., 1996; Moore, L.E. *et al*., 1996, 1997a). Both types of damage were induced, but clastogenicity predominated with high exposure to arsenic (Moore, L.G. *et al*., 1996, 1997a).

Apart from the pilot study of Ostrosky-Wegman *et al*. (1991), no induction of *HPRT* mutation was found in a further study of 15 male Chilean copper-roasting-plant workers (aged 24–66 years), who were categorized according to job type as being exposed to arsenic at low, medium or high levels. Their mean duration of employment in the factory was 43 months. The individual exposure was ascertained by analysing levels of arsenic in the urine. In the very highly exposed workers (internal dose, 260 µg/L arsenic in urine), no induction of *HPRT* mutations in peripheral lymphocytes was demonstrated. The authors concluded that the *HPRT* assay seems to have a low sensitivity for the detection of the genotoxicity of arsenic *in vivo* (Harrington-Brock *et al*., 1999).

Another study of 70 Chilean men with long-term exposure to 600 µg/L arsenic in drinking-water and 55 frequency-matched control subjects (15 µg/L arsenic in drinking-water) determined micronuclei in bladder cells (Biggs *et al*., 1997; Moore, L.E. *et al*., 1997a). Matching criteria were age, smoking status, time of local residence (average high exposure, 19.3 years), education and ethnicity. An exposure-related increase in the frequency of micronuclei was found in the exposure quintiles 2–4 (urinary arsenic,

54–729 µg/L), but not in the 5th quintile (urinary arsenic > 729 µg/L). The prevalence of centromer-positive micronuclei increased 3.1-fold in quintile 4 (95% CI, 1.4–6.6), and the prevalence of centromer-negative micronuclei increased 7.5-fold in quintile 3 (95% CI, 2.8–20.3), suggesting that chromosome breakage was the major cause of formation of micronuclei. An intervention study was carried out on a subset of 34 of the arsenic-exposed Chilean men of this investigation. The arsenic-contaminated drinking-water supply (600 µg/L) was changed to water containing 45 µg/L arsenic. After 8 weeks, the prevalence of micronuclei in bladder cells decreased from 2.63/1000 cells before the intervention to 1.80/1000 cells after the intervention for all individuals. The frequencies of micronuclei in exfoliated bladder cells had significantly decreased from 4.45/1000 cells before the intervention to 1.44/1000 cells after the intervention in smokers but not in nonsmokers (2.05/1000 cells versus 1.90/1000 cells), suggesting that the bladder cells of smokers could be more susceptible to genotoxic damage caused by arsenic (Moore, L.E. *et al*., 1997b).

The frequency of micronuclei in 12 Andean women and 10 children with lifetime current exposure to 200 µg/L arsenic in the drinking-water was compared with that in 10 women and 12 children exposed to 0.7 µg/L arsenic. Putative confounding variables such as smoking, consumption of alcohol and coca leaves were included in the evaluation. It was shown that the frequencies of micronuclei per 1000 binucleated cells in peripheral lymphocytes were significantly elevated in the arsenic-exposed groups as compared with controls (women, 41 versus 8.5; children, 35 versus 5.6, respectively) (Dulout *et al*., 1996). Moreover, the frequency of aneuploidy was significantly elevated (0.21% versus 0%; 12 exposed versus 17 controls). In contrast, the frequencies of sister chromatid exchange in the arsenic-exposed group were not affected (5.7 versus 5.5 per cell in exposed and control women and 4.4 versus 4.6 per cell in exposed and control children, respectively), nor were specific chromosome translocations.

Induction of sister chromatid exchange was found in peripheral lymphocytes of subjects after 20 years of exposure to arsenic in well-water (> 130 µg/L) in Argentina (Lerda, 1994). Putative exposures to other genotoxic compounds were reported to be taken into account in the study. The mean frequency of sister chromatid exchange was 10.50 per cell in exposed men and women (282 nonsmokers) versus 7.50 per cell in 155 control subjects (volunteer men and women) drinking water that contained less than 20 µg/L arsenic for more than 20 years. Exposed subjects were significantly older than the control group (mean age, 56.71 versus 38.90). In a further evaluation, to homogenize the age of the exposed group, participants older than 50 years were excluded from the analysis. In the younger subset, no correlation between sister chromatid exchange and sex, or sister chromatid exchange and age was found. Sister chromatid exchange was induced by concentrations as low as 100 µg/L arsenic for the younger subset. Moreover, the arsenic content in drinking-water was associated with the frequency of sister chromatid exchange in both sexes but was not affected by sex. [The Working Group noted that the value of the study is reduced because the statistical evaluation was not reported in detail. Moreover, arsenic in urine was quantified by an insensitive colorimetric method of analysis.]

4.4.2 *Experimental systems* (see Table 30 for details and references)

(*a*) *In-vitro studies*

The methylated forms of trivalent arsenic are the only arsenic species that cause DNA damage *in vitro* (Mass *et al.*, 2001; Nesnow *et al*., 2002).

Arsenic (sodium arsenite) did not induce tryptophan revertants in *Escherichia coli* or ouabain- or 6-thioguanine-resistant mutants in Chinese hamster lung (V79), Chinese hamster ovary or Syrian hamster embryo cells (Lee *et al.*, 1985a). Moreover, induction of SOS repair by sodium arsenite was not detected in *E. coli PQ37*. However, sodium arsenite and sodium arsenate were mutagenic in mouse lymphoma L5178Y cells, inducing trifluorothymidine-resistant mutants.

Sodium arsenite induced a significantly increased frequency of sister chromatid exchange in Chinese hamster ovary and Syrian hamster embryo cells. Sodium arsenate was one order of magnitude less potent in inducing sister chromatid exchange than sodium arsenite. It induced the formation of micronuclei in Chinese hamster ovary and V79 cells in the cytokinesis-block micronucleus test using cytochalasin B as well as in the absence of cytochalasin B in V79 cells and also induced chromosomal aberrations in mammalian cells.

Sodium arsenite significantly elevated the frequency of sister chromatid exchange and significantly enhanced micronucleus formation in isolated human peripheral lymphocytes as well as in whole blood after cytokinesis block through cytochalasin B. It induced chromosomal aberrations as chromatid gaps, fragmentation, endoreduplication and chromosomal breaks in human leukocytes, lymphocytes and primary umbilical cord fibroblasts. Moreover, induction of aneuploidy was observed in human peripheral lymphocytes treated with sodium arsenite *in vitro*, suggesting that this clastogenic agent may exhibit some weak aneuploidogenic properties.

There is some evidence that human, mouse and rat leukocytes are more sensitive to the induction of micronuclei after treatment with arsenite than guinea-pig leukocytes (Peng *et al*., 2002). This difference in the induction of micronuclei by arsenic could not be explained by a species-dependent variability in arsenite methylation. The leukocytes of all four species were able to ethylate arsenic but there was no clear correlation between the ability to methylate arsenic and the induction of micronuclei.

In assays with mouse lymphoma L5178Y cells, arsenate (As^V), MMA^V and DMA^V induced mutations at the *Tk* locus, chromosomal aberrations and micronuclei. Arsenobetaine, the major arsenic compound in seafood, did not induce neoplastic transformation in mouse fibroblast BALB/3T3 cells.

Significant increases in chromosomal aberrations were induced in human umbilical cord fibroblasts by arsenate, MMA^V, DMA^V, trimethylarsine oxide, arsenosugar, arsenocholine, arsenobetaine and tetramethylarsonium iodide. The higher potency of induction of chromosomal aberrations by DMA^V in comparison with MMA^V was probably caused by contamination of DMA^V sample by inorganic arsenic (Eguchi *et al*., 1997). Nevertheless,

Table 30. Genetic and related effects of arsenic and arsenic compounds

Test system	Results[a]	Dose[b] (LED or HID)	Reference
Arsenate (As^{V})			
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus, *in vitro*	+	10	Moore, M.M. *et al.* (1997)
Gene mutation, Syrian hamster embryo cells, ouabain resistance, *in vitro*	–	31	Lee *et al.* (1985a)
Gene mutation, Syrian hamster embryo cells, 6-thioguanine resistance, *in vitro*	–	31	Lee *et al.* (1985a)
Sister chromatid exchange, Syrian hamster embryo cells *in vitro*	+	3.1	Lee *et al.* (1985a)
Micronucleus formation, mouse lymphoma L5178Y cells *in vitro*	+	10	Moore, M.M. *et al*. (1997)
Chromosomal aberrations, mouse lymphoma L5178Y cells *in vitro*	+	10	Moore, M.M. *et al*. (1997)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	20	Lee *et al.* (1985a)
Cell transformation, Syrian hamster embryo cells	+	5	Lee *et al*. (1985a)
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	5	Oya-Ohta *et al*. (1996)
Chromosomal aberrations, human lymphocytes *in vitro*	–	1	Nordenson *et al.* (1981)
Chromosomal aberrations, human leukocytes *in vitro*	+	2.25 (0.6 ppm as As)	Nakamuro & Sayato (1981)
Arsenite (As^{III})			
Escherichia coli, gene mutation (tryptophan revertant selection) *in vitro*	–	3250	Rossman *et al*. (1980)
Escherichia coli, *LacZ* gene induction (SOS chromotest) *PQ37 in vitro*	–	105	Lantzsch & Gebel (1997)
Gene mutation, Chinese hamster ovary cells, ouabain resistance, *in vitro*	–	0.65	Rossman *et al.* (1980); Lee *et al.* (1985b)
Gene mutation, Chinese hamster ovary cells, 6-thioguanine resistance, *in vitro*	–	13	Rossman *et al.* (1980)
Gene mutation, Chinese hamster ovary cells, 6-thioguanine resistance, *in vitro*	–	1.3	Lee *et al.* (1985b)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus, *in vitro*	+	1	Moore, M.M. *et al*. (1997b)
Gene mutation, Syrian hamster embryo cells, ouabain resistance, *in vitro*	–	1.3	Lee *et al.* (1985a)
Gene mutation, Syrian hamster embryo cells, 6-thioguanine resistance, *in vitro*	–	1.3	Lee *et al.* (1985a)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	+	0.65	Lee *et al.* (1985b)

Table 30 (contd)

Test system	Results[a]	Dose[b] (LED or HID)	Reference
Sister chromatid exchange, Syrian hamster embryo cells *in vitro*	+	0.1	Lee *et al.* (1985a)
Micronucleus formation, Chinese hamster ovary cells *in vitro*	+	5.21[c]	Wang *et al.* (1997)
Micronucleus formation, Chinese hamster V79 cells *in vitro*	+	0.325	Gebel (1998)
Micronucleus formation, mouse lymphoma L5178Y cells *in vitro*	+	1.5	Moore, M.M. *et al*. (1997)
Chromosomal aberrations, mouse lymphoma L5178Y cells *in vitro*	+	1.5	Moore, M.M. *et al*. (1997)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	0.8	Lee *et al*. (1985a)
Cell transformation, Syrian hamster embryo cells	+	0.20	Lee *et al*. (1985a)
Sister chromatid exchange, human lymphocytes *in vitro*	+	0.03	Gebel *et al.* (1997); Rasmussen & Menzel (1997); Nordenson *et al.* (1981)
Micronucleus formation, human lymphocytes *in vitro*	+	0.06	Schaumlöffel & Gebel (1998)
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	0.5	Oya-Ohta *et al*. (1996)
Chromosomal aberrations, human lymphocytes *in vitro*	+	0.09	Nordenson *et al.* (1981)
Chromosomal aberrations, human leukocytes *in vitro*	+	0.31	Nakamuro & Sayato (1981)
Aneuploidy, human lymphocytes *in vitro*	+	0.4	Eastmond & Tucker (1989)
Aneuploidy, human lymphocytes *in vitro*	+	0.31 ng/mL	Ramírez *et al.* (1997)
Single-cell gel assay (comet), Swiss albino mouse leukocytes *in vivo*	+	0.13 mg/kg po	Saleha Banu *et al.* (2001)
LacZ gene mutation, Muta^{TM} mouse lung, kidney, bladder, bone marrow *in vivo*	–	7.6 mg/kg ip × 5	Noda *et al.* (2002)
Micronucleus formation, BALB/c mouse bone marrow *in vivo*	+	10 mg/kg (24 h) or 0.5 mg/kg (30 h) ip	Deknudt *et al.* (1986)
Micronucleus formation, BALB/c/CBA/C57BL mouse bone marrow *in vivo*	+	5 mg/kg ip	Tinwell *et al.* (1991)
Micronucleus formation, B6C3F1 mouse bone marrow *in vivo*	+	5 mg/kg po × 4	Tice *et al.* (1997)

Table 30 (contd)

Test system	Results[a]	Dose[b] (LED or HID)	Reference
Micronucleus formation, Muta^{TM}mouse peripheral blood reticulocytes *in vivo*	+	7.6 mg/kg ip × 5	Noda *et al.* (2002)
Chromosomal aberrations, Swiss mouse bone marrow *in vivo*	+	0.1 mg/kg sc × 4	Roy Choudhury *et al.* (1996)
Chromosomal aberrations, Swiss mouse bone marrow *in vivo*	+	2.5 mg/kg po	Biswas *et al.* (1999)
Dominant lethal mutation, Balb/c mouse *in vivo*	–	5 mg/kg ip	Deknudt *et al.* (1986)
Monomethylarsonic acid (MMA^{V})			
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus, *in vitro*	+	2500	Moore, M.M. *et al*. (1997)
Micronucleus formation, mouse lymphoma L5178Y cells *in vitro*	+	4000	Moore, M.M. *et al*. (1997)
Chromosomal aberrations, mouse lymphoma L5178Y cells *in vitro*	+	4000	Moore, M.M. *et al*. (1997)
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	196	Oya-Ohta *et al*. (1996)
Dimethylarsinic acid (DMA^{V})			
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus, *in vitro*	+	5000	Moore, M.M. *et al*. (1997)
Micronucleus formation, mouse lymphoma L5178Y cells *in vitro*	–	10 000	Moore, M.M. *et al*. (1997)
Chromosomal aberrations, mouse lymphoma L5178Y cells *in vitro*	+	8000	Moore, M.M. *et al*. (1997)
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	96.6	Oya-Ohta *et al*. (1996)
DNA strand break, ICR CD-1 mouse lung *in vivo*	+	1500 mg/kg	Yamanaka *et al.* (1989); Yamanaka & Okada (1994)
DNA strand break, ICR CD-1 mouse liver, kidney and spleen *in vivo*	–	1500 mg/kg	Yamanaka *et al.* (1989); Yamanaka & Okada (1994)
LacZ gene mutation, Muta^{TM} mouse lung, kidney, bladder, bone marrow *in vivo*	–	10.6 mg/kg ip × 5	Noda *et al.* (2002)
Micronucleus formation, Muta^{TM} mouse peripheral blood reticulocytes	–	10.6 mg/kg ip × 5	Noda *et al.* (2002)
Aneuploidy, CD-1 mouse bone marrow *in vivo*	+	300 mg/kg ip	Kashiwada *et al.* (1998)

Table 30 (contd)

Test system	Results[a]	Dose[b] (LED or HID)	Reference
Trimethylarsine oxide (TMAO)			
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	503	Oya-Ohta *et al*. (1996)
Arsenocholine			
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	4950	Oya-Ohta *et al*. (1996)
Arsenobetaine			
Cell transformation, mouse BALB/3T3 cells	–	89	Sabbioni *et al*. (1991)
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	1958	Oya-Ohta *et al*. (1996)
Tetramethylarsonium iodide			
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	4978	Oya-Ohta *et al*. (1996)
Arsenosugar (2′,3′-Dihydroxypropyl-5-deoxy-5-dimethylarsinoyl-β-D-riboside)			
Chromosomal aberrations, primary human umbilical cord fibroblasts *in vitro*	+	4860	Oya-Ohta *et al*. (1996)
Methylarsonous acid (MAs^{III})			
Single-cell gel (comet) assay, human lymphocytes *in vitro*	+	2.12[c]	Mass *et al*. (2001)
Dimethylarsinous acid ($DMAs^{III}$)			
Single-cell gel (comet) assay, human lymphocytes *in vitro*	+	1.22[c]	Mass *et al*. (2001)

[a] +, positive; –, negative; without exogenous metabolic system

[b] LED, lowest effective dose; HID, highest ineffective dose unless otherwise stated; in-vitro tests, μg/mL; in-vivo tests, mg/kg bw/day; po, oral; ip, intraperitoneal

[c] Estimated from graph in paper

Eguchi *et al.* (1997) had shown that pure DMA^V but not MMA^V had induced tetraploids in Chinese hamster V79 cells.

MMA^{III} and DMA^{III} were investigated using human lymphocytes in the single-cell gel assay. At low micromolar doses, these methylated trivalent arsenicals showed a comet-like tail corresponding to DNA damage. In this study, neither As^{III}, As^V, nor the methylated pentavalent arsenicals produced significant nicking, strand breaks or alkali labile lesions in DNA compared with the methylated trivalent arsenicals.

Both hypomethylation and hypermethylation of DNA were associated with exposure to arsenic in cultures of human lung A549 cells and in human kidney UOK cells (Zhong & Mass, 2001). This could be consistent with the proposal that changes in DNA methylation can activate some genes and repress others in response to exposure to arsenite.

(*b*) *In-vivo studies*

Swiss Albino mice administered arsenic trioxide (also called arsenite, As^{III}) orally showed a significantly increased DNA tail-length in leukocytes in the single-cell gel (comet) assay at the lowest dose tested.

Induction of DNA single-strand breaks was detected in the lung, but not in liver, kidney or spleen of ICR (CD-1) mice 12 h after oral administration of DMA^V. The DNA damage was completely repaired after a further 12-h interval.

No significant mutagenesis of the *lacZ* gene was observed in male transgenic Muta™ mouse lung, kidney, bladder or bone marrow after five daily intraperitoneal injections of arsenite (As^{III}) or DMA^V. However, arsenite significantly increased the frequencies of micronucleated reticulocytes in peripheral blood, whereas DMA^V had no effect. [The Working Group noted that, in comparison with other studies using DMA^V, the dose tested was more than one order of magnitude lower.]

Sodium arsenite dissolved in water and administered intraperitoneally to CBA, BALB/c and C57BL mice resulted in a significant induction of micronuclei in the polychromatic erythrocytes, as did oral administration to $B6C3F_1$ mice. Potassium arsenite tested only in C57BL mice was also positive in the micronucleus test in polychromatic erythrocytes. Arsenic sulfide (called orpiment) did not induce micronuclei to any quantifiable extent, presumably because of its low solubility and bioavailability, a reflection of elevated blood levels of arsenic in orpiment-treated animals. After oral or subcutaneous administration of sodium arsenite for either 1, 6 or 30 consecutive days, elevated frequencies of chromosomal aberrations were found in the bone-marrow cells of Swiss albino mice.

Significantly elevated numbers of aneuploid cells were detected in bone-marrow cells of ICR (CD-1) mice treated intraperitoneally with DMA^V.

In an assay to detect point mutations caused by arsenic, virgin C57BL/6J mice and female metallothionein knock-out null mice ($MT^{-/-}$) were exposed to drinking-water containing 500 µg/L arsenic for up to 26 months (Ng *et al*., 2001). Nine of 12 (75%) virgin C57BL/6J and 8/11 (72.72%) $MT^{-/-}$ mice developed one or multiple mutations in exon 5 of the *p53* gene. The most prominent mutation (mutation hot spot) appeared in codon 163

of exon 5, in 9/12 (75%) and 10/14 (71.4%) of the tissues tested in C57BL/6J and $MT^{-/-}$ mice, respectively.

C57BL/6J mice fed methyl-deficient diets were administered arsenite in the drinking-water at doses of 0, 2.6, 4.3, 9.5 or 14.6 mg/kg bw per day for 130 days. Arsenite treatment increased genomic hypomethylation in a dose-dependent manner and reduced the frequency of methylation at several cytosine sites within the promoter region of the Ha-*ras* gene (Okoji *et al*., 2002).

Co-mutagenicity/co-genotoxicity of arsenic

Trivalent arsenic was demonstrated to act as a synergistic co-mutagen in combination with many genotoxic agents including ultraviolet (UV) light.

For instance, when Chinese hamster ovary cells were treated simultaneously with UV light and sodium arsenite, chromatid and chromosomal aberrations as well as *Hprt* mutations were increased synergistically. An additive effect in the induction of sister chromatid exchange was observed with a combined treatment of low doses of UV and As^{III} but not with a combined treatment of higher doses of UV and arsenic (Lee *et al.*, 1985b). Treatment of Chinese hamster ovary cells with sodium arsenite after incubation with the DNA-alkylating agent methyl methanesulfonate also enhanced clastogenicity and *Hprt* mutagenicity synergistically (Lee *et al*., 1986a). However, pretreatment with sodium arsenite resulted in a reduction in the mutagenicity of methyl methanesulfonate. Furthermore, post-treatment of Chinese hamster ovary cells with sodium arsenite was shown to increase UV- and alkylating agent-induced chromosomal aberrations (Huang *et al*., 1986) and the clastogenicity of DNA-cross-linking agents (Lee *et al*., 1986b). In the presence of sodium arsenite, γ-ray-induced chromosomal aberration frequency was potentiated in human peripheral lymphocytes (Jha *et al*., 1992). In human UV-irradiated VH16 fibroblasts, micronuclei (but not sister chromatid exchange) were induced synergistically by post-treatment with sodium arsenite (Jha *et al*., 1992). According to the authors, the lack of synergistic effect on UV-induced sister chromatid exchange in this study may be because sodium arsenite was washed off before the cells were seeded for division.

4.5 Mechanistic considerations

Several different mechanisms of arsenic-induced carcinogenicity have been proposed, and the trivalent species are implicated in most of these mechanisms (National Research Council, 1999, 2001; Simeonova & Luster, 2000; Kitchin, 2001; Hughes, 2002). It should be noted, however, that the trivalent species are formed *in vivo* after exposure to pentavalent arsenic. Methylated trivalent arsenic is more toxic, and genotoxic, than trivalent inorganic arsenic; in contrast, methylated pentavalent arsenic is less toxic, and genotoxic, than pentavalent inorganic arsenic.

4.5.1 *Genotoxicity*

Arsenic induces chromosomal aberrations, micronuclei, aneuploidy, endoreduplication and gene amplification. These may play a role in the genomic instability that can result from treatment with arsenic. Arsenic appears to have little if any ability to induce point mutations (National Research Council, 1999, 2001). The methylated trivalent molecules of arsenic are potent forms for the induction of DNA damage in cells *in vitro*, and they are the only forms of arsenic that cause DNA breakage *in vitro*, a reaction that is mediated by reactive oxygen species (Yamanaka & Okada, 1994; Nesnow *et al*., 2002; Kitchin & Ahmad, 2003).

4.5.2 *Altered DNA repair*

Trivalent arsenic (As^{III}) inhibits nucleotide-excision repair of UVC-induced DNA damage in human fibroblasts by interacting with distinct steps of the repair process. It impaired the incision step at low concentrations and the ligation step at higher concentrations (Hartwig *et al.*, 1997).

As^{III} inhibits several DNA-repair enzymes including DNA ligases I and II (Li & Rossman, 1989; Lee-Chen *et al.*, 1992), and zinc-finger proteins bearing covalent disulfide linkages seem to be potential targets of this metal. The activity of PARP, one of the zinc-finger DNA-repair enzymes, is inhibited in a human T-cell lymphoma-derived Molt-3 cell line and HeLa cells by low concentrations of arsenic (5 μM and 10 nM, respectively) (Yager & Wiencke, 1997; Hartwig *et al.*, 2003). However, other zinc-finger DNA-repair enzymes such as mammalian xeroderma pigmentosum group A protein and bacterial formamido-pyrimidine-DNA glycosylase are not inhibited by As^{III} (Asmuss *et al.*, 2000).

4.5.3 *Induction of oxidative stress*

Exposure to arsenic results in the generation of reactive oxygen species both *in vitro* and *in vivo*. There is evidence that these may be involved in the DNA-damaging activities of As^{III}, MMA^{III} and DMA^{III}. Arsenic species, particularly DMA^{III}, release iron from ferritin (Ahmad *et al.*, 2000); this free iron can produce reactive oxygen species via Fenton and/or Haber-Weiss type reactions. Reactive oxygen species are detected in human–hamster hybrid cells exposed to arsenite (As^{III}) (Liu, S.X. *et al.*, 2001) and in ϕX174 DNA incubated *in vitro* with MMA^{III} or DMA^{III} (Nesnow *et al.*, 2002). They are also involved in stress responses that may alter DNA and gene expression. For example, 8-OHdG formation and cyclooxygenase Cox-2 expression, most commonly used as a marker for the evaluation of oxidative DNA damage, are increased in the urinary bladder cancers of rats treated with dimethyl arsenite (Wei *et al.*, 2002). The DMA^{III} produced *in vivo* in the urine of rats treated with DMA^{V} (Cohen *et al.*, 2002) and subsequent generation of reactive oxygen species may be important factors in the arsenic-induced bladder cancer observed in these animals (Wei *et al.*, 2002).

4.5.4 *Altered DNA methylation*

The alteration of DNA methylation by arsenic may also play a role in the development of cancer. In-vitro and in-vivo studies indicate that the carcinogenicity of arsenic may be mediated by alterations in the methylation status of DNA either by hypermethylation or hypomethylation (Mass & Wang, 1997; Zhao *et al.*, 1997; Okoji *et al.*, 2002).

4.5.5 *Cell transformation*

Arsenic induces cell transformation in Syrian hamster embryo cells, BALB/3T3 cells and in the rat liver cell line TRL1215. Inoculation of the latter cells into nude mice gave rise to malignant tumours (fibrosarcoma and metastases to the lung) (Lee *et al.*, 1985a; Bertolero *et al.*, 1987; Zhao *et al.*, 1997).

4.5.6 *Altered cell proliferation*

Increased cell proliferation has been demonstrated directly or indirectly in various experimental systems after exposure to arsenic (Germolec *et al.*, 1997; Kitchin, 2001; Hughes, 2002).

Increases in ODC activity, a biomarker of cell proliferation, have been observed in the kidney or liver of rats treated with arsenic (Yamamoto *et al.*, 1995; Brown & Kitchin, 1996). Stimulation of cell proliferation had been shown in normal human epidermal keratinocytes treated *in vitro* by arsenic (Germolec *et al.*, 1997).

Hyperplasia has been observed in the bladder of rats treated with DMA^V (Cohen *et al.*, 2002).

4.5.7 *Altered cell signalling*

Arsenic stimulates the activity of Jun kinases, which belong to the mitogen-activated protein kinase family, and increases the DNA binding of transcriptional factor AP-1. Arsenic also induces the expression of proto-oncogenes such as C-*JUN*, C-*FOS*, C-*MYC* and tumour growth factor-α (Cavigelli *et al.*, 1996; Germolec *et al.*, 1998; Simeonova *et al.*, 2000; Chen *et al.*, 2001). A reduction in p53 protein levels concomitant with an increase in mdm_2 protein levels were also observed in a keratinocyte (HaCaT) cell line treated with arsenic. The disruption of *P53-*MDM_2 loop-regulating cell-cycle arrest as a model for arsenic-related skin carcinogenesis has been proposed (Hamadeh *et al.*, 1999).

4.5.8 *Altered steroid receptor binding and gene expression*

Arsenic inhibited steroid binding to glycocorticoid receptors but had no effect on the binding of ligands to androgen, estrogen, mineral corticoid or progesterone receptors. This specific inhibition may provide a method of using arsenic to block glucocorticoid receptors selectively in assays of the progesterone receptor content of breast cancer tissues

(Lopez *et al.*, 1990). In MCF-7 cells, arsenite blocked the binding of estradiol to oestrogen receptor-α (ER-α) (Stoica *et al.*, 2000).

Moreover, arsenic inhibited expression of ER-α but had no effect on expression of ER-β in breast cancer cell lines (Chen *et al.*, 2002). Thus, the authors concluded that the role of arsenic in the expression of ER-α provides a novel therapeutic approach for ER-α-positive breast cancer (Chen *et al.*, 2002).

4.5.9 *Gene amplification*

Arsenic enhanced the amplification of the dihydrofolate reductase (*DHFR*) gene in mouse 3T6 cells and gene amplification has been suggested as a possible mechanism of the carcinogenicity of arsenic (Lee *et al.*, 1988).