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# Arsenobetaine and arsenocholine: two marine arsenic compounds without embryotoxicity

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**The embryotoxicity of carboxymethyl(trimethyl)arsonium bromide [arsenobetaine, $(CH_3)_3As^+CH_2COO^-$] and of 2-hydroxyethyl(trimethyl)arsonium bromide [arsenocholine, $(CH_3)_3As^+CH_2CH_2OH)Br$] was explored. Sprague–Dawley rat embryos with intact yolk sacs were removed on day 11 of gestation and grown in a culture medium for 24 h in the presence and absence of rat liver (S-9) homogenate. Solutions of arsenobetaine or arsenocholine in dimethyl sulfoxide [DMSO, $(CH_3)_2SO$] (0.03 $cm^3$) were added to the media to achieve concentrations of 20 $\mu$g arsenic compound per $cm^3$ of medium. After 24 h the circulation and heart beat were monitored (indicator of embryolethality); in addition the crown-to-rump lengths were measured and the neural structures (somites) and limb buds observed (indicator of embryotoxicity). No evidence for embryotoxicity or embryolethality was found in the absence or the presence of S-9. These results indicate that arsenobetaine, the most common arsenic compound found in seafood at concentrations from several micrograms to several hundred micrograms arsenic per gram, lacks subacute and acute prenatal toxicity.**

**Keywords: Arsenobetaine, arsenocholine, embryotoxicity, rat embryo, postimplantation**

## INTRODUCTION

Arsenic has the reputation of being toxic, carcinogenic, mutagenic, and teratogenic. Whereas certain arsenic compounds undoubtedly cause acute and chronic toxicoses in animals and man,[1,2] bring forth mutations,[3,4] elicit teratogenic effects,[4,5] and may even induce tumors,[3,6,7] statements attributing these effects to 'arsenic' are scientifically unsound and are spreading misinformation. Arsenic forms many inorganic and organic compounds.[8] Each of these compounds has unique physical, chemical, and biological properties. For instance, arsenite, an inorganic species containing trivalent arsenic, is much more toxic according to $LD_{50}$ values than methylated arsenic compounds such as methylarsonic acid and dimethylarsinic acid, which are examples of organic arsenic compounds containing pentavalent arsenic.[9] Exposure limits expressed in terms of total arsenic will certainly protect animal and human populations from overexposure if these limits are set sufficiently low. However, such low limits might be unnecessary and economically damaging. If the US arsenic limit for drinking water (50 $\mu$g $dm^{-3}$) were applied to seafood as 50 $\mu$g $kg^{-1}$, all seafood would be unfit for consumption; seafood often has arsenic concentrations at 1000 times this limit.[10] Should arsenic become an essential element for man – animal studies[11] support this contention – exposure limits for arsenic set too low could lead to widespread arsenic deficiencies with unknown consequences.[12]

Arsenic exposure limits should ideally be set in terms of specific arsenic compounds or mixtures of arsenic compounds. To arrive at such limits, toxicological properties of arsenic compounds must be known. During the investigations of their toxicological properties, the possibility must be kept in mind that arsenic compounds can be metabolized by organisms. Arsenic compounds formed by metabolic reactions from inorganic arsenic compounds generally are less toxic than their precursors. The arsenic cycle in the marine environ-

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ment[13] links inorganic arsenite and arsenate with methylarsonic acid [$CH_3AsO(OH)_2$], dimethylarsinic acid [$(CH_3)_2AsO(OH)$], trimethylarsine oxide [$(CH_3)_3AsO$], tetramethylarsonium salts [$(CH_3)_4As]^+$,[14] arsenocholine, and arsenobetaine. Arsenobetaine, the trimethyl(carboxymethyl)arsonium cation, appears to be the most common arsenic compound in marine organisms. Frequently, more than 90% of the total arsenic in marine organisms is present in the form of arsenobetaine; total arsenic concentrations may reach 100 mg $kg^{-1}$.[10] Arsenobetaine administered to mice orally at a dose of 400 mg As $kg^{-1}$ body weight did not appear to be toxic.[15] Arsenocholine, the trimethyl (2-hydroxyethyl)arsonium cation, detected at low levels in shrimp,[16,17] fish, and shellfish,[18] is probably the precursor of arsenobetaine. Mice, rats, and rabbits have been shown to be able to convert arsenocholine to arsenobetaine.[19]

To date, no studies have been conducted to ascertain the prenatal toxicity of arsenobetaine and arsenocholine. We exposed postimplantation rodent embryos to these two organic arsenic compounds to investigate whether they have embryotoxic properties.

## EXPERIMENTAL

### Chemicals

Arsenocholine [trimethyl(2-hydroxyethyl)arsonium bromide][20] and arsenobetaine [trimethyl(carboxymethyl)arsonium bromide][21] were prepared and purified according to literature procedures. Waymouth's 752/1 media, Hanks' Balanced Salt Solution (HBSS), penicillin and streptomycin were obtained from Gibco Inc. (Grand Island, NY, USA) and glucose 6-phosphate and NADPH from Sigma Chemical Company (St Louis, MO, USA). Microsomal (S-9) hepatic supernatants, prepared from male rats pretreated with Aroclor, were purchased from Microbiological Associates (Bethesda, MD, USA).

### Animals

Nulliparious, female, Sprague–Dawley rats (225–250 g) were obtained from Harlan–Sprague–Dawley Labs (Houston, TX, USA). The rats were bred after having been kept on a daily 12-h light, 12-h dark cycle. Conception was monitored by aspirating vaginal fluids and checking them for the presence of sperm. The day on which sperm was found was considered day one of gestation.

### Embryo cultures

For each compound to be tested, a culture medium (15 $cm^3$) was prepared from Waymouth's 752/1 medium (7.5 $cm^3$), frozen rat serum (7.5 $cm^3$) prepared from the sacrificed rats, penicillin (1500 units), and streptomycin (1.5 mg).[22] To test compounds in the presence of bioactivating enzymes, sodium glucose 6-phosphate (21 mg) and S-9 (0.225 $cm^3$) were added to the culture medium. Almost saturated dimethyl sulfoxide (DMSO) solutions of arsenobetaine (10.1 mg arsenobetaine $cm^{-3}$ DMSO) and arsenocholine (10.0 mg arsenocholine $cm^{-3}$ DMSO) were prepared. Aliquots (0.03 $cm^3$) of these solutions were mixed with the culture medium (15 $cm^3$) on a roller culture apparatus (Wheaton, Inc., Millville, NJ, USA). Control embryos were grown in media of identical composition. Instead of the DMSO solution of arsenobetaine or arsenocholine (0.03 $cm^3$), pure DMSO (0.03 $cm^3$) was added to the control media.

On day 11 of gestation (day-10 embryos) at least nine pregnant rats were etherized. Blood for the preparation of serum was collected by needle-puncture of the abdominal aorta. The uteri were excised and placed into HBSS kept at room temperature. Each embryo was

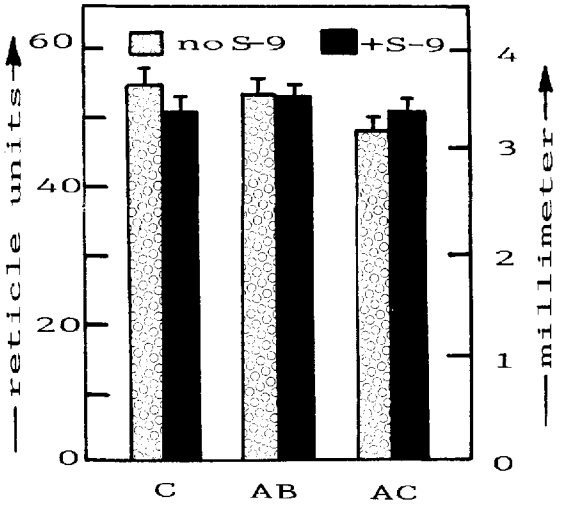


**Figure 1** Crown-to-rump length of rat embryos after 24 h in media containing arsenobetaine or arsenocholine (20 $\mu$g arsenic compound/$cm^3$ medium) in the absence or presence of S-9. C, control; AB, arsenobetaine in the medium; AC, arsenocholine in the medium.

surgically separated under a stereomicroscope. The decidua and Reichert's membrane were then removed to obtain each embryo still enclosed by its yolk sac. The embryos were then washed with HBSS. Groups of nine embryos (all from different rats) were placed into loosely capped 125-$cm^3$ glass culture bottles containing 15 $cm^3$ of the appropriate culture media. The bottles were placed on a roller culture apparatus rotating at 40 rpm. The apparatus was kept in a humidified 5% $CO_2$-in-air incubator maintained at 37°C. After 24 h the embryos were examined under a stereozoom microscope fitted with a measuring reticule for viablity, abnormalities, and growth. Embryoviability, used as an index of embryolethality, was determined by the presence or absence of active yolk sac circulation and heart beat. Crown-to-rump length, somite count, and limb-bud development were monitored as indices of embryotoxicity.

## RESULTS AND DISCUSSION

In the USA, 560 000 infant deaths, spontaneous abortions, stillbirths, and miscarriages and 200 000 birth defects are reported annually.[23] Epidemiological studies associate 25% of these birth defects and conceptus/neonate deaths to genetic causes,[24] 10% to known teratogens and prenatal toxins, such as thalidomide, rubella and radiation, and the remaining 65% to unknown causes. Evidence is accumulating that man-made chemicals and chemicals 'naturally' present in the environment are important etiological agents responsible for the birth defects and prenatal deaths currently blamed on unknown causes.

Arsenic compounds, commonly believed to be much more potent than they actually are, have been frequently investigated with respect to their effects on man and animals. However, little is known about the effects during the prenatal stages during which a developing organism is very sensitive toward chemical influences. Because large groups of people are exposed to arsenic through ingestion of seafood, arsenic compounds concentrated in seafood deserve to be investigated to check whether these compounds cause birth defects or prenatal death, to identify the mixtures of chemicals that potentiate the prenatal toxicity of individual arsenic compounds, and to explore the molecular mechanisms that are responsible for the prenatal effects of specific arsenic compounds.

Whole-animal studies are too costly and lengthy to provide proper indicators for human prenatal toxic potential and accurate assessments of additive and synergistic properties of suspect compounds. Short-term, *in vitro* assays that quickly identify potent toxins and toxin mixtures must be used to achieve results in a timely manner. The effects of arsenobetaine, the most common arsenic compound in marine animals, and of arsenocholine, a potential precursor of arsenobetaine, on ten-day-old rat embryos were investigated. Six groups of nine embryos each with intact yolk sacs were formed from the embryos taken from different rats. These embryos were kept in a medium with rat serum as the base. Dimethyl sulfoxide, the least embryotoxic of the common carriers (corn oil, trioctanoin) in such toxicity studies, was used as solvent for arsenocholine and arsenobetaine. The maximal volume of DMSO that can be added to the growth medium without increasing background levels of toxicity and abnormalities is 2 $\mu$l $cm^{-3}$ of medium. Therefore, 30 $\mu$l of DMSO were added to the control medium and 30 $\mu$l of DMSO solutions of arsenobetaine or arsenocholine to the other media.

At concentrations of 10 $\mu$g arsenobetaine or arsenocholine per $cm^3$ of medium, the exposed embryos developed as well as the control embryos. When 0.03 $cm^3$ of almost saturated solutions of arsenobetaine or arsenocholine in DMSO were added to the media, no adverse effects on the embryos could be observed. The average crown-to-rump length (nine embryos), a measure of embryonic growth, after 24 h of exposure, was 3.63 mm for the controls, 3.50 mm for the group exposed to arsenobetaine, and 3.37 mm for the group exposed to arsenocholine without S-9, the mix of bioactiving enzymes (Fig. 1). The differences in embryonic growth are not statistically significant. Arsenocholine and arsenobetaine do not impair growth in the absence of S-9 (Fig. 2).

Somites are progenitor structures from which components of the spinal column and central nervous system develop. Previous studies exposing rats and rat embryos to macrocyclic antibiotics,[25] polycyclic aromatic hydrocarbons,[26] and toxic plant alkaloids[27] established a correlation between *in vitro* inhibition of somite development and spinal abnormalities occurring after *in vitro* exposure to the compounds. Therefore, the somite structures of the embryos exposed to arsenobetaine and arsenocholine were monitored as indicators of the neurotoxic potential of the arsenic compounds.

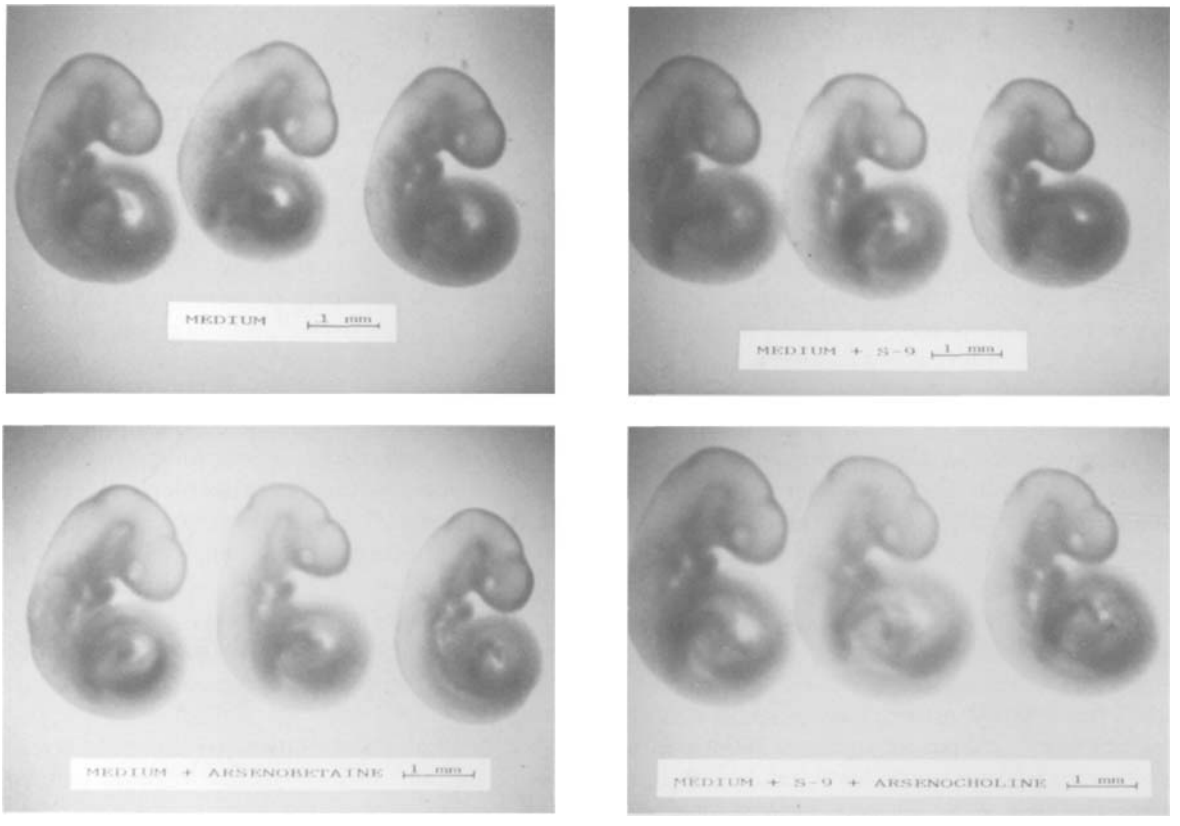


**Figure 2** Rat embryos (three each; day 11 of gestation) after 24 h of growth in the medium, in the medium + S-9 (controls), in the medium containing 10 $\mu$g arsenobetaine/cm$^3$ medium, and in the medium + S-9 containing 10 $\mu$g arsenocholine/cm$^3$ medium.

No significant differences in somite development between control embryos and exposed embryos were observed (Table 1).

The circulation of red blood cells through the yolk sacs and the embryonic heartbeat were monitored as indicators of direct embryolethality. The development of limb buds, the progenitor structures of rodent limbs, was also checked. These indicators showed no significant differences between control embryos and embryos exposed to arsenobetaine or arsenocholine (Table 2). The two arsenic compounds, therefore, do not possess any direct embryotoxicity *in vitro*.

In separate experiments embryos were exposed to arsenobetaine and arsenocholine in the presence of rat liver homogenate (S-9) prepared from Aroclor-induced rats. This liver homogenate contains several oxidative and reductive enzymes that are concentrated in the endoplasmic reticulum of the liver and other organs and serve to transform exogenous xenobiotic compounds into water-soluble derivatives for urinary or fecal excretion.[28] However, many xenobiotic compounds are converted by these enzymes to very reactive intermediates that damage macromolecules, such as proteins and DNA, and cause cells to die. Thus, addition of S-9 to embryo cultures exposes embryonic cells also to derivatives of arsenobetaine and arsenocholine that may be formed by enzyme-promoted reactions. No significant difference in crown-to-rump lengths was observed between the control embryos (3.37 mm) and the embryos exposed to arsenobetaine/S-9 (3.46 mm) or arsenocholine/S-9 (3.37 mm) (Fig. 1). Somite development (Table 1), heartbeat, yolk sac circulation, and limb bud development (Table 2) were also not affected by the addition of S-9 to the embryo cultures (Fig. 2).

The experiments exposing cultured rat embryos to arsenobetaine and arsenocholine failed to show any toxic effects at concentrations of 20 $\mu$g of arsenobetaine bromide (5.8 $\mu$g As) cm$^{-3}$ or 20 $\mu$g of arsenocholine bromide (6.1 $\mu$g As) cm$^{-3}$ in the

**Table 1** Effect of arsenocholine and arsenobetaine on embryonic somite development

| Embryo culture | Average somite number[a] |
|---|---|
| Control | 20.4 ± 1.2 |
| Control + S-9 | 20.2 ± 0.9 |
| Arsenocholine | 21.4 ± 1.6 |
| Arsenocholine + S-9 | 20.7 ± 1.5 |
| Arsenobetaine | 21.1 ± 2.0 |
| Arsenobetaine + S-9 | 18.9 ± 1.4 |

[a]Average number of somites per embryo ± standard deviation from nine embryos.

**Table 2** Effect of arsenocholine and arsenobetaine on embryonic ultrastructure development

| | Number of embryos | | |
|---|---|---|---|
| Treatment | With heartbeat | With yolk sac circulation | With limb bud development |
| Control | 9/9 | 7/9 | 9/9 |
| Control + S-9 | 9/9 | 9/9 | 9/9 |
| Arsenocholine | 9/9 | 9/9 | 9/9 |
| Arsenocholine + S-9 | 9/9 | 8/9 | 9/9 |
| Arsenobetaine | 9/9 | 9/9 | 9/9 |
| Arsenobetaine + S-9 | 9/9 | 9/9 | 9/9 |

culture medium. These results suggest that prenatal toxic effects are unlikely to occur in humans after consumption of seafood that naturally contains arsenobetaine at concentrations comparable[29] with those in the medium in which the rat embryos grew. The inability of arsenobetaine and arsenocholine to pass through the membrane of the yolk sac, and thus to keep the immediate environment of an embryo low or free of arsenic, might account for the observed lack of toxic effects. However, this scenario is unlikely, because in experiments on embryo systems with other organic arsenic compounds and inorganic arsenic compounds (e.g. arsenite) toxic effects were clearly evident.[30] Arsenobetaine[15] and arsenocholine[19] are known to cross easily the intestinal tract–blood barrier in mice, rats, rabbits, and man[31] and to be excreted via the kidneys. Therefore, these arsenic compounds will very likely cross the yolk sac membrane also. To verify this hypothesis, arsenic would have to be determined in the liquid within the yolk sac and within the embryos. Because of the small volumes involves ($<0.1$ cm$^3$), such determinations are extremely difficult if not impossible unless radioactive tracers are used. Grown animals excrete arsenobetaine unchanged[15] and convert arsenocholine to arsenobetaine.[19] Whether pregnant rats or rat embryos metabolize arsenobetaine and arsenocholine differently is not known. Previous investigations[32] demonstrated that pregnant animals biotransform and excrete xenobiotic compounds differently than non-pregnant animals. Additional experiments should be performed to determine whether microsomal homogenates from tissues of pregnant rats metabolize arsenobetaine and arsenocholine. If biotransformation occurs, the effect of the metabolites on rat embryos must be explored.

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