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Glycerylphosphorylarsenocholine and phosphatidylarsenocholine in yelloweye mullet (*Aldrichetta forsteri*) following oral administration of arsenocholine

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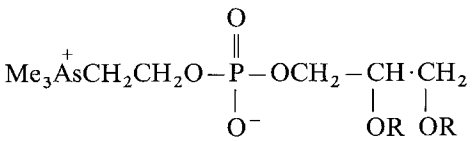
Summary. The novel arsenical glycerylphosphorylarsenocholine has been isolated from yelloweye mullet (*Aldrichetta forsteri*) following oral administration of arsenocholine. Ether-soluble arsenic was shown to be present as phosphatidylarsenocholine.
Key words. Glycerylphosphorylarsenocholine; phosphatidylarsenocholine; arsenocholine; arsenobetaine.

Although arsenobetaine (**1**) is recognised as the major form of arsenic in marine animals, the biosynthetic pathway for this compound is not fully understood[1]. Evidence suggests that arsenobetaine is accumulated by marine animals via the food chain rather than directly from seawater[2]. Arsenic is present in marine macroalgae, at levels comparable to those found in marine animals, primarily as dimethylarsinylribosides, and these algal arsenic compounds are a possible source of arsenobetaine[3]. Arsenocholine (**2**) is a likely intermediate in the conversion of dimethylarsinylribosides to arsenobetaine[4].

(1)

(2)

In a recent study[5] on the accumulation of arsenic compounds by the yelloweye mullet (*Aldrichetta forsteri*) we showed that fish readily metabolised administered arsenocholine to arsenobetaine. Other metabolites of arsenocholine were a weakly basic water-soluble compound and some ether-soluble arsenic, but the small quantities precluded further examination. Additional experiments have now provided larger quantities of these two arsenic fractions and we here report the isolation and identification of glycerylphosphorylarsenocholine (**3**) from yelloweye mullet fed arsenocholine. Ether-soluble arsenic (lipid arsenic) was shown to be present as phosphatidylarsenocholine (**4**) by the isolation and identification of compound 3 following alkaline hydrolysis of the lipids.



(3) R = H
(4) R = $CO(CH_2)_nCH_3$

Juvenile yelloweye mullet were fed meat (beef) dosed with arsenocholine as previously described[5]. Yelloweye mullet were used as experimental fish because of their ease of maintenance in aquaria. Furthermore, the small amount of native arsenic in juvenile yelloweye mullet was insignificant relative to the quantity accumulated during experimentation. Arsenocholine bromide (163 mg ≡ 50

mg As) was administered to 10 fish (average wt 15.5 g containing native arsenic at ~ 0.4 µg As/g) in 8 daily meals. Twenty-four hours after their final meal, the fish, now at ~ 120 µg As/g, were extracted to yield insoluble (0.5 mg As), water-soluble (18.1 mg As) and ether-soluble (0.4 mg As) fractions. The water-soluble fraction was applied to a column of Dowex 50 H^+ resin (water as eluate) and arsenic was monitored in the eluent by graphite furnace atomic absorption spectrometry. Some of the arsenic (0.32 mg) was shown to be weakly basic by its slow elution from the column with water (5 × void volume). Arsenic which was retained on the column, previously shown to be arsenobetaine [5], was not further examined. The weakly basic arsenic compound was purified by thin layer chromatography (TLC) on cellulose developed in butan-1-ol/ acetic acid/water, 60:15:25 (system A, R_f 0.30) and propan-1-ol /NH_3, 7:3 (system B, R_f 0.37), followed by gel permeation chromatography (Sephadex G-15/water) to yield a syrup (~ 0.8 mg, 0.23 mg As). This syrup was identified as the novel glycerylphosphorylarsenocholine (**3**) by a comparison of its NMR spectra (^1H and ^{13}C), field desorption mass spectrum and chromatographic properties (Dowex 50, TLC systems A and B) with those of a synthetic specimen. Racemic glycerylphosphorylarsenocholine (**3**), prepared by a modification of the method used to prepare glycerylphosphorylcholine [6], was obtained as a syrup which contained: C, 30.6; H, 6.2. $C_8H_{20}AsO_6P$ requires: C, 30.2; H, 6.3%. FD-MS, *m/z* 319 $(M + H)^+$, 165, 147. ^1H NMR (300 MHz, D_2O) δ 1.96, s, Me_3As; 2.78, broadened triplet, *J* 6.0 Hz, $AsCH_2$; 3.61, dd, 1 H, J_{gem} 11.7, J_{vic} 6.0 Hz, 3.68, dd, 1 H, J_{gem} 11.7, J_{vic} 4.5 Hz, $HOCH_2$; 3.84–3.96, m, $O_3POCH_2CHOHCH_2OH$; 4.25–4.31, two overlapping triplets, *J* 5.8, *J* 6.0 Hz, $AsCH_2CH_2OPO_3$. ^{13}C NMR (75.5 MHz, D_2O) δ 7.6, s, Me_3As; 26.7, d *J* 9 Hz, $AsCH_2$; 60.2, d *J* 6 Hz, $AsCH_2CH_2OPO_3$; 62.2, s, CH_2OH; 66.6, d *J* 6 Hz, $O_3POCH_2CHOHCH_2OH$; 70.8, d *J* 9 Hz, CHOH.

The possibility that arsenic-containing phospholipids were present in the ether-soluble fraction was investigated by examining the water-soluble products from hydrolysis of the lipid arsenic. Thus after the method of Chadha [7] (used to prepare glycerylphosphorylcholine from phosphatidylcholine) tetraethylammonium hydroxide (0.4 mMol) in methanol (1 ml) was added to a portion of the ether-soluble fraction (168 mg, 21 µg As) in ether (50 ml). The solution darkened and a brown syrup separated within minutes. After 30 min the mixture was partitioned between ether/water whereby > 95% of the arsenic was found in the aqueous layer. Neutralisation (HCl) of the aqueous layer followed by chromatography on a column of Dowex 50 H^+ resin (water as eluate) gave a weakly basic fraction (7.4 mg, 22 µg As). Purification of the weakly basic arsenical by TLC (cellulose, systems A and B) and gel permeation chromatography (Sephadex G-15/water) yielded a product (< 0.2 mg, 20 µg As) identified as glycerylphosphorylarsenocholine (**3**) by a comparison of its chromatographic properties and ^1H NMR spectrum with those of the synthetic sample.

The virtually quantitative yield of glycerylphosphorylarsenocholine (**3**) on alkaline hydrolysis of the lipid arsenic was taken as evidence for the presence of phosphatidylarsenocholine (**4**) in the ether-soluble fraction. This arsenic-containing phospholipid is likely to occur because arsenocholine replaces choline in enzyme systems for the biosynthesis of phosphatidylcholine [8]. The probable precursors in this biosynthesis, phosphorylarsenocholine and cytidine diphosphoarsenocholine, were not found in this study but they may have been present at low levels. Glycerylphosphorylarsenocholine (**3**) presumably arises from metabolism of phosphatidylarsenocholine (**4**) within the fish tissues. Almost 40% of the total arsenic ingested as arsenocholine (**2**) was retained by the fish. The major metabolite was arsenobetaine (**1**) constituting > 90% of the retained arsenic; glycerylphosphorylarsenocholine (**3**) and phosphatidylarsenocholine (**4**) each accounted for ~ 2%.

Although arsenocholine is a likely intermediate in the biosynthesis of arsenobetaine from dimethylarsinylribosides, it is yet to be rigorously identified in a marine organism. Its presence, incorporated into lipids, has been suggested [9] for a unicellular alga and a crustacean exposed experimentally to high levels of arsenate in seawater. Reports of arsenocholine in shrimp [10] have been questioned [11] and require confirmation. Results of feeding arsenocholine to fish [5] suggest that it would be rapidly metabolised in marine food chains and unlikely to accumulate to appreciable levels. Products of arsenocholine metabolism would be predominantly arsenobetaine, with smaller quantities of glycerylphosphorylarsenocholine (**3**) and phosphatidylarsenocholine (**4**). It remains to be established whether compounds **3** and **4** occur naturally in marine organisms. Certainly for some marine animals, ether-soluble arsenic accounts for a considerable proportion of the total arsenic [12]. Although a phosphatidylarsinylriboside has been identified in lipids from a brown alga [13], the form of lipid arsenic in marine animals remains unknown. The present study indicates that phosphatidylarsenocholine (**4**) is a possible candidate.

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D-Amino acids in mouse tissues are not of microbial origin

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Summary. Neutral free D-amino acid contents in the serum, kidney, liver, brain, small intestine and urine in germ-free mice and those in specific pathogen-free mice were compared. No significant difference was found. This strongly suggests that the free D-amino acids which were shown to be present in mice in our previous work [1, 2] did not originate from the enteric microbial flora.
Key words. D-Amino acid; microflora; germ-free; mice.

It has long been believed that the naturally occurring amino acids have an L-configuration in mammals [3]. Only D-aspartic acid is known to accumulate in metabolically stable proteins in humans, as a result of in vivo racemization [4–6]. However, we have observed considerable amounts of free D-amino acids (DAA) in plasma from patients with renal diseases [7], and in the kidney [2] of a mutant mouse lacking D-amino acid oxidase, using a sensitive enzymic method for estimating neutral DAA [1, 8]. Free DAA were also detected in normal human plasma [7], and in normal mouse kidney, liver, brain, heart, lung, thymus and serum [1]. Recently, the presence of free DAA has been confirmed by high performance liquid chromatography in the serum and in kidney extracts from a mutant mouse. D-isomers of alanine, proline, serine and glutamine were detected (Nagata et al., unpubl. obs.).

Hoeprich [3] resolved the paradox that *Mycobacterium tuberculosis* was unsusceptible to D-cycloserine in the treatment of experimental tuberculosis in guinea pigs and mice, although it was susceptible to the drug on testing in vitro, by detecting D-alanine, an antagonist to the antibiotic action of D-cycloserine, in the serum of these animals. He thus provided the first documentation of the occurrence of a free D-amino acid in the blood of mammalian species. However, his conclusion was that the D-alanine in guinea pigs and mice was probably from an exogenous source, most likely from the enteric microflora, for no D-alanine had been detected in the serum of guinea pigs and mice from germ-free colonies. His view is still the prevailing one.

In order to investigate the source of DAA, we measured free DAA levels in the serum, kidney, liver, brain, small intestine and urine from bacteria-free mice, and compared them with those from normal control mice. The result was in contrast to that of Hoeprich, i.e., no difference was observed between the two kinds of mice. Therefore, the source for DAA is not likely to be the microbial flora of the intestine. An interpretation of the discrepancy is given under 'Results and discussion'.

Materials and methods

Male germ-free (GF) mice (ICR, 6 weeks old) and age- and sex-matched specific pathogen-free (SPF) mice of the same strain were obtained from Japan Clea (Tokyo). They were maintained in the Animal Care Center of Sapporo Medical College for 15 days as follows. Each animal was housed alone in an autoclave-sterilized plastic cage with a mesh floor, in order to prevent the mice from having access to feces, because mice may be coprophagous, and the feces of an SPF mouse contain many bacteria. D-alanine and D-glutamic acid are known to be components of the bacterial cell wall [9]. The cages were kept in a vinyl isolator, which was freshly sterilized. The mice were fed autoclaved NIH-improved type chow (Oriental Yeast, Tokyo) which included no supplemental DAA, and supplied with autoclaved water. The GF mice were confirmed to be axenic until the last day of breeding, by culture tests of specimens collected from the diet chow, drinking water and feces, and the inner surfaces of the cages, on days 0, 7, 11 and 15. The specimens were cultured in thioglycollate medium for 7 days at 37 °C as well as at room temperature, and in glucose-peptone medium for 10 days at room temperature. The SPF mice were shown by the culture tests to have been infected with both aerobic and anaerobic bacteria, and eumycetes.

Blood was collected from axillary vessels under anesthesia with ether, after 16–19 h starvation, and the serum was separated by centrifugation after clotting at room temperature. The kidney, liver, small intestine and brain