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Arsenic Mononucleotides. Separation by High-Performance Liquid Chromatography and Identification with Myokinase and Adenylate Deaminase†

Rosario Lagunas,* David Pestaña, and José C. Diez-Masa

ABSTRACT: The spontaneous formation of arsenic mononucleotides has been detected in mixtures of arsenate and inosine or adenosine or its deoxy analogues. These compounds have been separated by high-performance liquid chromatography and identified by their behavior in the presence of myokinase and adenylate deaminase. The nucleoside 5′-arsenates are formed preferentially to the 2′- and 3′-arsenate analogues. All arsenic nucleotides detected showed similar kinetic and equilibrium constants of formation: about 8×10^{-4} M^{-1} s^{-1} and 2×10^{-3} M^{-1}, respectively. These values are several orders of magnitude greater than those of their phosphoric analogues. The adenosine 5′-arsenate was able to substitute for 5′AMP in the reaction of myokinase and adenylate deaminase. The substitutions of the 2′- or 3′-hydrogen for hydroxyl groups in the ribose moiety of this compound slightly affected its suitability as substrate for myokinase but had drastic effect in the case of adenylate deaminase. The half-life of the arsenic nucleotides, at pH 7.0 and 25 °C, ranged from 30 to 45 min. The lability of these compounds is increased during catalysis with myokinase. Results on the reaction mechanism of myokinase with adenosine 5′-arsenate indicate that the mixed-anhydride analogue to ADP, adenosine 5′-(arsenate phosphate), is not detected either because it is not formed in the reaction with this enzyme or because it is rapidly hydrolyzed.

The occurrence of labile arsenic esters in cells supplied with arsenate was postulated (Braunstein, 1931) to explain the observation that arsenate decreased alcoholic fermentation in yeast (Harden & Young, 1906). Evidence supporting this hypothesis was obtained when the existence of sugar–arsenic esters was demonstrated in mixtures of sugars and arsenate (Lagunas, 1968; Long & Ray, 1973; Lagunas, 1980). The formation of arsenic nucleotides has been the subject of intensive investigation in mitochondria because the uncoupling effect of arsenate on the oxidative phosphorylation was explained through the formation of a labile intermediate of this type (Ernster el al., 1967; Bertagnolli & Hanson, 1973). However, although some evidence was reported for the oc-

† From the Instituto de Enzimología y Patología Molecular del CSIC, Facultad de Medicina de la UAM, Madrid-34, Spain, and the Instituto de Química Orgánica del CSIC, Juan de la Cierva 3, Madrid-6, Spain. *Received July 8, 1983.* Supported by Fondo Nacional para la Investigación Científica y Técnica.

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currence of a compound with properties such as those expected for an arsenic ester (Chan et al., 1969), only recently, the formation of an arsenic analogue of ATP, the ADP arsenate, has been demonstrated with mitochondrial particles under phosphorylating conditions (Gresser, 1981).

We noticed, during experiments with myokinase in reaction mixtures containing adenosine or its deoxy analogues and arsenate, the spontaneous formation of arsenic mononucleotides able to substitute for 5'AMP in the reaction catalyzed by this enzyme. Since, to our knowledge, these findings had not been previously reported, we have studied in this work the nature of these compounds, as well as their kinetic, thermodynamic, and biochemical properties. For this purpose, techniques of high-performance liquid chromatography (HPLC) have been developed that allowed separation of various arsenic nucleotides, as well as their identification by their ability to be used as substrates by myokinase and adenylate deaminase. The results indicate that the nucleoside 5'-arsenates are formed preferentially to the 2'- and 3'-arsenate analogues. All these arsenic nucleotides showed similar kinetic and equilibrium constants of formation whereas they showed important differences with respect to their phosphoric analogues. The reaction of adenosine 5'-arsenate (5'AMAs)[1] with myokinase was studied with [U-^{14}C]adenosine. The results indicate that the mixed-anhydride analogue to ADP, adenosine 5'-(arsenate phosphate) (AMAsP), either was not formed or was rapidly hydrolyzed to 5'AMAs and orthophosphate (P_i).

Materials and Methods

Reagents. Enzymes, coenzymes, and substrates were obtained from Sigma (St. Louis, MO) or from Boehringer (Mannhein, West Germany). [U-^{14}C]Adenosine was from the Radiochemical Center (Amersham, United Kingdom). All other reagents were of analytical grade.

Spontaneous Formation of Arsenic Mononucleotides. Mixtures of sodium arsenate at pH 7.0 and adenosine or its deoxy analogues, at the indicated concentrations, were incubated at 25 °C for the indicated time periods. In the experiments with [U-^{14}C]adenosine, 35 nmol (500 mCi/mmol) was lyophilized and dissolved in 50 μL of a mixture containing 1 M arsenate, pH 7.0, and 10 mM cold adenosine. The formation of arsenic nucleotides was analyzed enzymatically and by HPLC at different times of incubation as described below.

Analysis of 5'AMP and of the Arsenic Nucleotides with Myokinase. Aliquots of the incubation mixtures described above or of a solution of 5'AMP were added to an assay mixture containing 50 mM imidazole, pH 7.0, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM PEP, 1 mM ATP, and 0.15 mM NADH. Aliquots of 10 units of pyruvate kinase, 10 units of lactate dehydrogenase, and 50 units (18 μg) of rabbit muscle myokinase were used as auxiliary enzymes. The reaction was followed by monitoring the oxidation of NADH. Controls of the oxidation of NADH due to the presence of free adenosine or deoxyadenosine and arsenate were carried out by adding appropriate amounts of these compounds without previous incubation. A Gilford 2400 spectrophotometer was used.

Analysis of 5'AMP and of the Arsenic Nucleotides with Adenylate Deaminase. A 20-μL aliquot of a mixture of 1 M arsenate, pH 7.0, and 10 mM adenosine incubated for 1 h at 25 °C or appropriate aliquots of a solution of 5'AMP were added to 1 mL of an assay mixture containing 50 mM imidazole, pH 7.0, and 0.1 mM KCl. The reaction was started by addition of 4 μg of rabbit muscle adenylate deaminase (about 0.5 unit), and changes of absorbances at 265 nm were measured. Controls of the changes due to the presence of free adenosine and arsenate were run in parallel and gave undetectable values.

Separation and Analysis of Nucleotides by Reversed-Phase Pair-Ion HPLC. A modification of the method of Pennings & van Kempen (1979) was used. An analytical column (150 × 4.6 mm) packed with LiChosorb RP-18, 5 μm (Merck, Darmstadt, West Germany), and coupled with a guard column (40 × 4.6 mm) packed with μBondapak C_{18} Corasil, 37–50 μm, was used. The solvent system consisted of 15 mM tetrabutylammonium bromide as counterion and 30 mM KH_2PO_4 in methanol–water at the proportions indicated in each case. The counterion and the KH_2PO_4 were dissolved in water, and after adjustment to pH 7.0 with 5 M KOH, methanol was added, and the mixture was filtered through a Millipore filter of 0.45-μM pore size. A liquid chromatograph (Waters Associates, Milford, MA) was used that consisted of a M-6000 pump, a Model Rheodyne 7125 injector, and a Model 440 absorbance detector. Aliquots of 20 μL of the samples, treated as described in each experiment, were injected into the column, and the analysis was carried out at room temperature, at a flow rate of 1 mL/min and a pressure of 1500 psi. The absorbance at 254 nm was monitored. In the experiments with [U-^{14}C]adenosine, fractions of 0.1 mL were collected, dissolved in a scintillation mixture containing Triton X-100, and counted in a Beckman LS-100 counter. When separation was achieved in the absence of counterion, the solvent system contained 30 mM KH_2PO_4, pH 7.0, dissolved in methanol–water (1:9 v/v).

Concentrations of the arsenic nucleotides have been calculated by assuming that the arsenic and phosphoric nucleotides show similar spectral constants. This assumption is based on the fact that the optical properties of the adenosine nucleotides are due to the adenosine moiety of the molecule and are independent of the presence or the absence of the phosphate and hydroxyl residues (Bock et al., 1956; Ness & Fletcher, 1960). The calculated values have been corrected for the hydrolysis that occurred during HPLC analysis by taking into account the elution time and the stability of these compounds. For this purpose, samples of the mixtures were diluted 20-fold with the eluents and incubated at 25 °C. Samples of these dilutions were chromatographed at designated intervals. The hydrolysis of arsenic nucleotides showed first-order kinetics, and the half-lives were calculated by the formula: half-life = 0.301/slope.

Calculation and Expression of Kinetic and Thermodynamical Constants. Symbols, units, and terminology used in this paper are those recommended by the Interunion Commission on Biothermodynamics (1976) and the Nomenclature Committee of IUB and Joint Commission on Biochemical Nomenclature (1980). Calculations of the kinetic and thermodynamic constants have been performed as described by Morris (1974).

Results

Spontaneous Formation of Adenosine 5'-Monoarsenates. During incubation of mixtures of arsenate and adenosine, a compound accumulated that substituted for 5'AMP in the reaction catalyzed by myokinase (Figure 1A). This accumulation did not occur when arsenate was replaced by phosphate. The extent of the reaction catalyzed by the enzyme was proportional to the volume of incubation mixture assayed

[1] Abbreviations: 5'AMAs, adenosine 5'-arsenate; HPLC, high-performance liquid chromatography; 2'd-5'AMAs, 2'-deoxyadenosine 5'-arsenate; 2'd-3'AMAs, 2'-deoxyadenosine 3'-arsenate; 3'd-5'AMAs, 3'-deoxyadenosine 5'-arsenate; 3'd-2'AMAs, 3'-deoxyadenosine 2'-arsenate; AMAsP, adenosine 5'-(arsenate phosphate); P_i, orthophosphate; 5'IMAs, inosine 5'-arsenate; PEP, phosphoenolpyruvate.

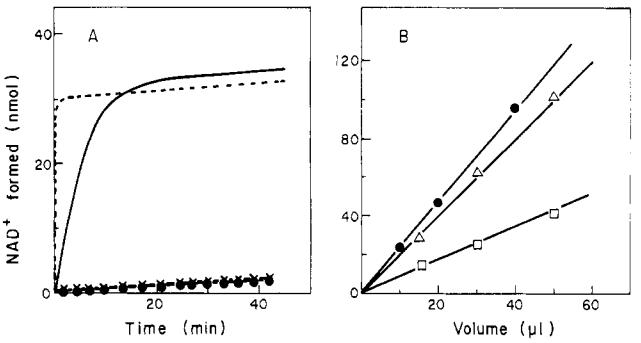

FIGURE 1: Detection by myokinase of the spontaneous formation of arsenic nucleotides. (A) Kinetics of the enzyme. A mixture (15 µL) containing 10 mM adenosine and 1 M arsenate (—) or phosphate (●), pH 7.0, incubated for 30 days at 25 °C, was added to 1 mL of an assay mixture containing 18 µg of myokinase. The activity of the enzyme was followed with the aid of pyruvate kinase and lactate dehydrogenase (see Materials and Methods). A mixture of arsenate and adenosine without previous incubation was also assayed in the absence (×) and in the presence (– –) of 15 nmol of 5′AMP. (B) Amount of nucleotides detected vs. volume of the aliquots assayed. Increasing aliquots of mixtures containing 1.0 M arsenate, pH 7.0, and 10 mM adenosine (●) or 2′-deoxyadenosine (□) or 3′-deoxyadenosine (△), incubated for 5 h at 25 °C, were added to the assay mixture. The amount of NAD^+ formed after 30 min of incubation in the presence of myokinase is shown.

(Figure 1B), to the concentration of adenosine and arsenate present in the mixture (results not shown), and to the incubation period (Figure 2A). When the mixtures were diluted, allowed to stay for increasing time periods, and again assayed with myokinase, a decrease of the accumulated compounds that followed first-order kinetics was observed (Figure 2B). These observations suggested that in aqueous mixtures of arsenate and adenosine an arsenic analogue of 5′AMP, 5′AMAs, is spontaneously formed until an equilibrium is reached according to the equation:

$$\text{adenosine} + \text{arsenate} \underset{k_{hydrol}}{\overset{k_{form}}{\rightleftharpoons}} \text{5′AMAs} + H_2O$$

In addition to 5′AMAs, 2′AMAs and 3′AMAs could also be formed in the mixture. However, 2′AMAs and 3′AMAs would not be detected by myokinase due to the specificity of this enzyme (Noda, 1973). Actually, in our hands, myokinase did not show activity in the presence of 1 mM 2′AMP and 3′AMP. When, instead of adenosine, 2′-deoxyadenosine or 3′-deoxyadenosine was present in the mixtures, activity of myokinase was also observed, indicating that 2′-deoxyadenosine 5′-arsenate (2′d-5′AMAs) and 3′-deoxyadenosine-5′-arsenate (3′d-5′-AMAs) were also spontaneously formed and that these compounds also behaved as substrates of the enzyme (Figures 1 and 2).

Possible Mechanisms of Reaction of Myokinase with 5′AMAs. Enzymatic measurements of the nucleoside 5′-arsenates could be achieved, in principle, by using the ability of these compounds to react in the presence of myokinase. However, no conclusive results could be obtained by this procedure because the reaction of myokinase with these compounds could follow, at least, two different mechanisms:

$$\text{5′AMAs} + \text{ATP} \longrightarrow \text{AMAsP} + \text{ADP} \quad (1)$$

$$\text{5′AMAs} + \text{ATP} \longrightarrow \text{5′AMAs} + P_i + \text{ADP} \quad (2)$$

Mechanism 1 postulates the formation of two reaction products, ADP and a mixed anhydride of arsenate and phosphate, AMAsP, at stoichiometrical amounts with respect to 5′AMAs. Formation of a mixed anhydride of this kind, the ADP arsenate, has been detected in experiments with submitochondrial particles (Gresser, 1981), and also, an analogue of ADP bearing an arsonomethyl group has been made by chemical synthesis (Webster, 1978).

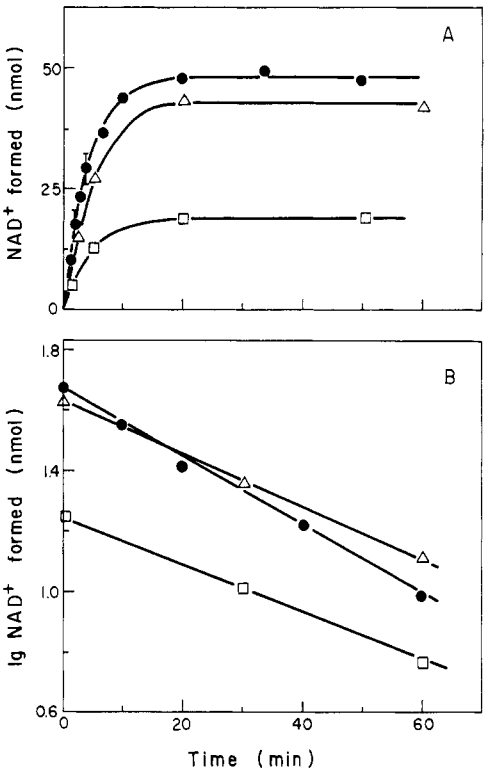

FIGURE 2: Kinetics of spontaneous formation and hydrolysis of arsenic nucleotides. (A) Spontaneous formation. Mixtures containing 0.5 M arsenate, pH 7.0, and 7.5 mM adenosine (●) or 2′-deoxyadenosine (□) or 3′-deoxyadenosine (△) were incubated at 25 °C. At the indicated times, 50 µL of these mixtures was added to 1 mL of the assay mixture described under Materials and Methods. The amount of NAD^+ formed after incubation for 30 min in the presence of myokinase is shown. The results are mean values of two or four experiments (those with standard deviation). (B) Spontaneous hydrolysis. Mixtures containing 0.5 M arsenate, pH 7.0, and 7.5 mM adenosine (●) or 2′-deoxyadenosine (□) or 3′-deoxyadenosine (△) were incubated for 2 h at 25 °C. A 0.1-mL aliquot of these mixtures was diluted to 1 mL with water and incubated at 25 °C. At the indicated times, 0.150 mL of the dilutions was assayed as above.

Mechanism 2 postulates that AMAsP either is not formed or is rapidly hydrolyzed to 5′AMAs and P_i. In this case, accumulation of only minute amounts of 5′AMAs in the mixtures of arsenate and adenosine would produce the great amounts of ADP detected in Figure 2A since the reaction would function as a cycle. In fact, in the absence of any other limiting factor, formation of ADP would continue as long as the stability of 5′AMAs allowed it.

Adenylate deaminase could also be suitable to determine 5′AMAs by its possible ability to catalyze the reaction:

$$\text{5′AMAs} \rightarrow \text{inosine 5′-arsenate (5′IMAs)} + NH_3$$

In fact, arsenic esters behave, in general, as good analogues of phosphoric esters in enzymatic reactions (Lagunas, 1968; Long & Ray, 1973; Jaffé et al., 1977; Lagunas, 1980). However, no activity of adenylate deaminase was observed with mixtures of arsenate and adenosine, indicating that either 5′AMAs is not a substrate of this enzyme or that 5′AMAs was present in the assays at undetectable concentration (see Materials and Methods).

Separation of Phosphoric and Arsenic Nucleotides by HPLC. The precedent results showed that the enzymatic procedures assayed were unsuitable for measuring arsenic

Table I: Separation of Nucleosides and Phosphoric Nucleotides by Reversed-Phase HPLC

	elution time (min)				
	with counterion at a methanol concn (%) of[b]				without counterion[c]
compd	25	20	15	10	
adenosine	2.1	2.4	3.2	4.2	6.9
2'dA		2.9		4.8	9.3
3'dA		3.1		5.0	7.8
inosine					2.9
5'AMP	2.5	3.3	4.5	6.7	3.8
2'AMP		5.5		17	
3'AMP		7.0		28	
5'IMP		2.7			2.5
ADP	3.9	5.6	10	21	
ATP	5.2	8.3	20	48	

[a] Samples of 20 μL of 10 μM solutions of the indicated nucleosides or nucleotides were injected to the column. [b] Eluent was 15 mM tetrabutylammonium bromide–30 mM KH_2PO_4, pH 7.0, in methanol-water at the indicated proportions (v/v). [c] Eluent was 30 mM KH_2PO_4, pH 7.0, in methanol-water (10:90, v/v).

nucleotides. They also showed that the lability of these compounds would not allow their separation by conventional chromatographic methods (Mangold, 1969) since, according to Figure 2B, arsenic nucleotides showed half-lives of only about 30 min. Techniques of HPLC seem, in principle, most appropriate to separate these compounds.

Rapid separation of phosphoric nucleotides can be achieved by HPLC with reversed-phase ion-pair techniques (Pennings & van Kempen, 1970; Brown et al., 1980). Since arsenic and phosphoric acids show similar p*K* values for their respective ions (Jenks & Rengenstein, 1970), rapid separation by these techniques could also be expected in the case of arsenic nucleotides. After several experiments, tetrabutylammonium was chosen as counterion, and phosphoric nucleotides were separated as shown in Table I. Among the mixtures of methanol–water assayed as solvent, the proportion 20:80 (v/v) gave the best results since, with the exception of 5'IMP, which eluted very close to the adenosine nucleosides, separation of nucleotides was achieved with good resolution in less than 9 min. Separation of 5'IMP from adenosine nucleosides was possible by eluting with a mixture without counterion (Table I).

When mixtures of arsenate and adenosine or 3'-deoxyadenosine were chromatographed in the presence (Figure 3A) or in the absence (Figure 3B) of counterion, in addition to the peaks corresponding to the free nucleosides, one small peak of absorption at 254 nm was detected, respectively. In the case of 2'-deoxyadenosine, two small peaks appeared. None of these peaks were detected if the mixtures were not previously incubated or if phosphate was substituted for arsenate. The size of these peaks increased with the concentration of the nucleosides and arsenate and with the incubation period of the mixtures until a maximum was reached in about 20 min. When these mixtures were diluted 20 times with either water or the eluents used in the HPLC techniques and the dilutions allowed to stay, the size of the peaks decreased following first-order kinetics similar to those enzymatically observed for the hydrolysis of the arsenic nucleotides (Figure 2B).

It became clear from these experiments that the small peaks resolved by HPLC behaved similarly to the arsenic nucleotides enzymatically detected, and identification of these peaks, as indicated in Figure 3, was achieved as described below.

Identification of Arsenic Nucleotides. Reaction with Myokinase. The reaction of myokinase with 5'AMAs was

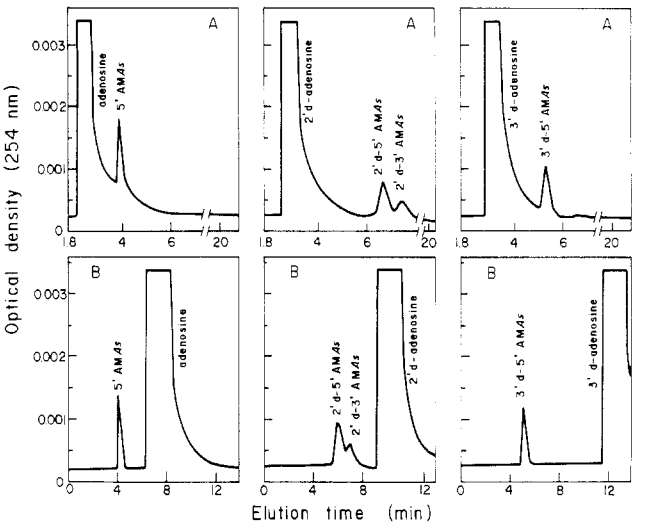

FIGURE 3: Separation of arsenic nucleotides by HPLC. Mixtures of 1.0 M arsenate, pH 7.0, and 10 mM adenosine or 2'-deoxyadenosine or 3'-deoxyadenosine incubated for 30 h at 25 °C were diluted 20-fold with water. Samples (20 μL) of these dilutions were immediately injected to the column and eluted as described under Materials and Methods with (A) 15 mM tetrabutylammonium bromide–30 mM HK_2PO_4, pH 7.0, in methane–water (20:80 v/v) or (B) 30 mM HK_2PO_4, pH 7.0, in methanol–water (10:90 v/v).

studied by HPLC with a mixture of [U-^{14}C]adenosine and arsenate. In these experiments, the small peak of absorption at 254 nm, spontaneously formed during incubation, showed radioactivity (Figure 4A). After 4 min in the presence of myokinase and ATP, the size of this peak decreased by 80% (Figure 4B), and after 10 min, it completely disappeared. Simultaneously, a decrease of ATP occurred that was accompanied by the formation of two peaks (Figure 4B) identified as 5'AMP and ADP by their elution times (Table I). These changes did not occur in the absence either of ATP (results not shown) or of the enzyme (Figure 4A). Addition of pyruvate kinase and PEP provoked a prompt disappearance of 5'AMP and ADP as well as the recovery of ATP to its initial value (Figure 4C).

As mentioned above, myokinase is specific for 5'AMP (Noda, 1973). Therefore, even if the 2'- and 3'-arsenic analogues were present, they would remain unchanged in the presence of the enzyme, and only the 5' analogue would be eventually transformed. The fact that the small radioactive peak disappeared by the action of myokinase in the presence of ATP (Figure 4B) suggests that this peak corresponds to 5'AMAs. This conclusion was supported by experiments with adenylate deaminase (see below).

In the conditions used in Figure 4, that is, in the presence of counterion, the elution times of the adenosine nucleotides increase with the electric charge of the molecules (Table I). Therefore, if 5'AMAs were transformed by myokinase into AMAsP as postulated by mechanism 1 (see above), the conversion of the radioactive peak of 5'AMAs into a peak of AMAsP with greater elution time would occur. The fact that such conversion was not detected (Figure 4B) suggests that, as postulated by mechanism 2 (see above), AMAsP either was not formed or was rapidly hydrolyzed to 5'AMAs and P_i. This conclusion is supported by comparing the amount of 5'AMAs added to the assay with the amount of ADP formed. Only 18 pmol of 5'AMAs was present in the chromatogram of Figure 4A. This value was calculated from the area of the corresponding peak (see Materials and Methods). Therefore, if mechanism 1 were operative, only 18 pmol of ADP would be formed in the reaction with the enzyme. The fact that more

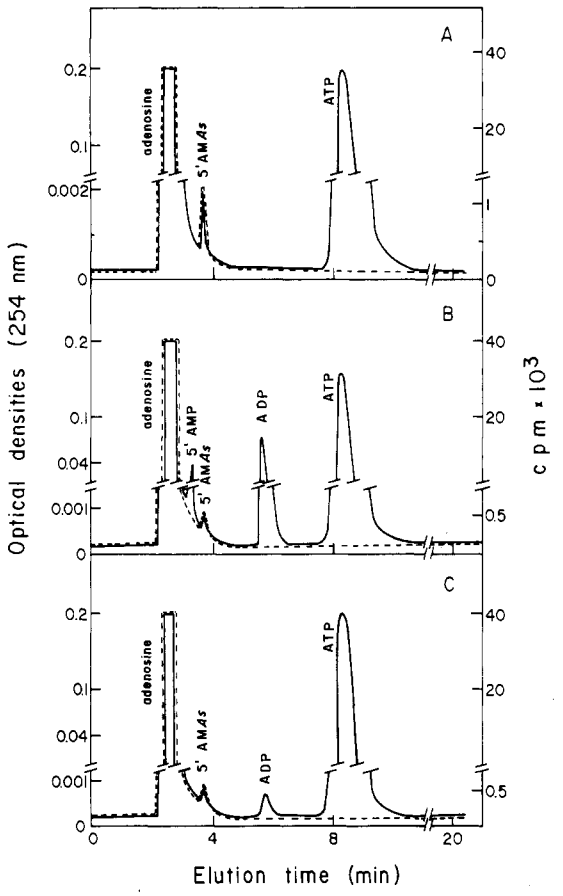

FIGURE 4: Reaction of 5'AMAs with myokinase. A total of 17.5 μCi of [U-^{14}C]adenosine, 500 mCi/mmol, was dissolved in 50 μL of 1 M arsenate and 10 mM adenosine. This mixture was incubated for 2 h at 25 °C. (A) Aliquots of 12 μL of this mixture were added to 0.168 μL of an assay mixture containing 0.1 M KCl, 5 mM $MgCl_2$, and 1 mM ATP. After incubation for 4 min, 20 μL of this mixture was injected to the column and eluted in the presence of counterion as in Figure 3, and the absorbance at 254 nm was monitored (continuous line). Elution fractions of 0.1 mL were collected and dissolved in the scintillation mixture, and the radioactivity was counted (dotted line). (B) The same as in (A) except that 30 μg of myokinase was present in the assay mixture. (C) The same as in (B) except that, after the 4 min of incubation in the presence of myokinase, 1 mM PEP and 10 units of pyruvate kinase were added, and the incubation was prolonged for 30 s. PEP gave a small peak with an elution time of 4.3 min that has been omitted for clarity reasons.

than 18 × 10^2 pmol of ADP was actually formed (Figure 4B) demonstrates that the reaction of myokinase with 5'AMAs functions as a cycle and, therefore, that mechanism 2 is the operative one.

The appearance of 5'AMP in the reaction of myokinase with 5'AMAs shown in Figure 4B could seem surprising. However, this appearance is easily explained by the activity of myokinase on the accumulated ADP as described by the equation:

$$2ADP \rightleftharpoons 5'AMP + ATP$$

This interpretation was demonstrated by the fact that, as the period of incubation with the enzyme increased, 5'AMP also increased at the expense of ADP until the equilibrium of this reaction was reached (results not shown).

The reaction of myokinase with a mixture of 3'-deoxyadenosine and arsenate showed similar characteristics to those shown in Figure 4, and the small peak (see Figure 3A) was identified as 3'd-5'AMAs by its decrease in the presence of the enzyme. In the case of 2'-deoxyadenosine, the reaction occurred at a lower rate, and identification of 2'd-5'AMAs could hardly be achieved because, under the conditions used, a decrease of only 15% of the greater of the two small peaks (see Figure 3A) was observed. This identification was better achieved by experiments with adenylate deaminase.

Table II: Kinetics and Thermodynamics of Arsenic Mononucleotides[a]

compd	k_{form} (M^{-1} s^{-1})[b]	K_c' (M^{-1})[c]	$\Delta G^{\circ\prime}$ (kcal mol^{-1})	half-life (min)[d]
5'AMAs	8.9×10^{-4}	2.2×10^{-3}	−3.6	30
5'IMAs	8.3×10^{-4}	2.4×10^{-3}	−3.6	37
2'd-5'AMAs	8.3×10^{-4}	1.9×10^{-3}	−3.7	40
3'd-5'AMAs	8.0×10^{-4}	2.0×10^{-3}	−3.8	45
2'd-3'AMAs	7.9×10^{-4}	1.1×10^{-3}	−4.0	40

[a] Mixtures containing 1 M arsenate and 10 mM inosine or adenosine or its deoxy analogues were incubated at 25 °C. [b] Samples of 20 μL of these mixtures were taken at intervals ranging from 1 to 5 min and chromatographed, after being diluted 20 times with water, as in Figure 3. Concentration of arsenic nucleotides was calculated from the size of the corresponding peaks, and the obtained values were corrected for the spontaneous hydrolysis that occurred during HPLC analysis as described in Materials and Methods. The correction factor ranged from 8 to 19%. The kinetic coefficients were calculated from the initial rates of formation of nucleotides as described by Morris (1974). [c] Equilibrium constants were calculated from data obtained from mixtures incubated at 25 °C for 1 h. After this period, formation of nucleotides had already reached equilibrium. [d] Samples of 50 μL of the mixtures incubated for 1 h were diluted 20-fold with water and incubated at 25 °C. Samples of 20 μL of these dilutions were chromatographed at 10-min intervals. Half-life was calculated as described under Materials and Methods. Mean values of two experiments are shown.

Reaction of Arsenic Nucleotides with Adenylate Deaminase. Adenylate deaminase is specific for 5'AMP (Zielke & Suelter, 1973), and therefore, as in the case of myokinase, it would only eventually transform the 5'-arsenic analogues. Actually, we could not detect activity with 1 mM 2'AMP or 3'AMP. In experiments with adenylate deaminase, elution was performed in the absence of counterion as in Figure 3B. When this enzyme was added to an incubated mixture of adenosine and arsenate, a small peak of inosine appeared as transformation product of the adenosine. Furthermore, 5'AMAs (see Figure 3B) was promptly transformed into a peak, presumably of inosine 5'-arsenate (5'IMAs), of similar size with an elution time of only 2.5 min. A similar peak of 5'IMAs was detected in incubation mixtures of inosine and arsenate. These results not only confirmed the identity of 5'AMAs but also demonstrated that this nucleotide is a good substrate of adenylate deaminase.

In the case of a mixture of 2'-deoxyadenosine, only the greater of the two small peaks (see Figure 3B) was affected by the presence of the enzyme. On this basis, we identified this peak as 2'-d-5'AMAs, its transformation product as 2'd-5'IMAs, which showed an elution time of 3.4 min, and the small peak that remained unchanged, tentatively, as 2'd-3'-AMAs. The results also suggested that 2'd-5'-AMAs behaved as a poor substrate of adenylate deaminase since 0.5 unit of this enzyme required as much as 20 min to transform about 10 pmol of this compound. The 3'd-5'AMAs behaved as substrate even worse since under similar conditions no transformation at all of this compound was observed (results not shown).

Kinetics, Thermodynamics, and Stability of Arsenic Nucleotides. The stability and the kinetic and thermodynamic constants of the spontaneous formation of the arsenic nucleotides, identified as described above, were calculated from data obtained by experiments of HPLC as shown in Table II. In water, these compounds showed half-lives that ranged from 30 to 45 min. These values decreased by 30% in the presence

of phosphate and methanol and by 50% when tetrabutylammonium was also present. In this later case, the observed half-lives ranged from 14 to 23 min.

Discussion

The results shown in this work indicate that the 5′-hydroxyl group of the ribose moiety of nucleosides reacts with arsenate giving rise to the spontaneous formation of the mononucleotides 5′-arsenate. No reactivity of the 2′-hydroxyl group was observed and, in the case of the 3′-hydroxyl group, the reaction was observed only when hydrogen substituted for the hydroxyl group in the 2′-position. These differences in the reactivity were probably due to the distinct accessibility of the hydroxyl groups to arsenate. However, with the exception of this steric aspect, it seems that the structure of the nucleosides scarcely influences the reaction. This is suggested by the fact that, when esterification did occur, the resulting mononucleotides showed similar kinetic and thermodynamic constants independent of whether they were derived from inosine, adenosine, or deoxyadenosine (Table II). By contrast, the esterification was greatly dependent on the acid reactant, whose nature affected not only the kinetics but also the equilibrium constant of the reaction. Our experiments suggest a rate coefficient of the spontaneous formation of the phosphoric mononucleotides, $k_{form} < 1 \times 10^{-9}$ M^{-1} s^{-1}, that is more than 10^{-5} times the value of their arsenic analogues. Furthermore, a Gibbs energy of hydrolysis for 5′AMP of $\Delta G° = -2.3$ kcal mol^{-1} has been reported (George et al., 1970) whereas values as great as -3.8 kcal mol^{-1} have been found for the arsenic derivatives (Table II). These data suggest important thermodynamic differences between arsenic and phosphoric nucleotides.

The 5′AMAs behaved as substrate of myokinase, and substitutions of hydrogen for hydroxyl groups in the ribose of this nucleotide slightly affected its suitability as substrate of this enzyme (Figures 1 and 2). 5′AMAs also behaved as a substrate of adenylate deaminase although, in this case, the substitutions of hydrogen led to more drastic effects. In fact, no activity of this enzyme with 3′d-5′-AMAs was detected in our experiments.

In the reaction of 5′AMAs with myokinase, the transfer of the phosphate residue from ATP to 5′AMAs was not observed. These results suggest that the arsenic analogue of ADP, AMAsP, either was not formed or was rapidly hydrolyzed to 5′AMAs and P_i (Figure 4). According to this and as suggested by the results of Figure 4, the reaction of myokinase with 5′AMAs would function as a cycle. Actually, this reaction would last as long as the stability of 5′AMAs would allow it. We have seen that the half-life of 5′AMAs was about 30 min (Table II), and accordingly, 5′AMAs would last in the assay for at least a couple of hours. However, it has been found that 5′AMAs disappeared in less than 10 min (Figure 4). This discrepancy between the expectations and the findings suggests that the mechanism of catalysis of myokinase increased the instability of the arsenic mononucleotides bound to the enzyme.

HPLC was shown to be a convenient technique for the separation of the arsenic mononucleotides. The great versatility of this technique (e.g., with respect to the composition, pH, temperature, and hydrophobicity of the eluent), combined with the relative high stability of these compounds (susceptible to enhancement under certain conditions), should permit their isolation for use as a tool in biochemical studies.

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Registry No. Arsenic, 7440-38-2; myokinase, 9013-02-9; adenylate deaminase, 9025-10-9; adenosine, 58-61-7; inosine, 58-63-9; 2′dA, 958-09-8; 3′dA, 73-03-0; 5′AMP, 61-19-8; 2′AMP, 130-49-4; 3′AMP, 84-21-9; 5′IMP, 131-99-7; ADP, 58-64-0; ATP, 56-65-5; 5′AMAs, 88392-05-6; 5′IMAs, 88392-06-7; 2′d-5′AMAs, 88392-07-8; 3′d-5′AMAs, 88392-08-9; 2′d-3′AMAs, 88392-09-0.

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