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# ELUCIDATION OF THE 2-AMINOETHYLPHOSPHONATE BIOSYNTHETIC PATHWAY IN TETRAHYMENA PYRIFORMIS

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SUMMARY. The biosynthetic reaction pathway leading to the natural product, 2-aminoethylphosphonate in Tetrahymena pyriformis has been elucidated. Incubation of [$^{32}$P]PEP and [$^{14}$C]PEP with T.pyriformis cellular homogenate fortified with $Mg^{2+}$ and alanine/pyridoxal phosphate, yielded 2-aminoethylphosphonate as the minor reaction product (2-5% yield) and phosphoglycerate and pyruvate plus orthophosphate as the major products. Inclusion of thiamine pyrophosphate in the reaction mixture increased the yield of 2-aminoethylphosphonate by a factor of 10. Incubation of phosphonoacetaldehyde or phosphonopyruvate in the cellular homogenate also provided 2-aminoethylphosphonate. The cellular homogenate catalyzed the transformation of phosphonoacetaldehyde to 2-aminoethylphosphonate in an ca. 80% yield. However, the maximum yield of 2-aminoethylphosphonic acid obtained by use of phosphonopyruvate was only 15%. The major reaction pathways induced by treatment of phosphonopyruvate with the cellular extract involved its competitive conversion to PEP and pyruvate plus orthophosphate.


Organophosphates metabolized by biological systems are usually oxygen esters, diesters, or anhydrides of phosphoric acid. In recent years members of a different class of organophosphates namely, phosphonates have been discovered in bacteria, unicellular plants, lower fungi and in animals belonging to several phyla, including man (1). By virtue of their P-C bond the phosphonates are set apart from the more familiar forms of naturally occuring organophosphates.

The mechanisms by which P-C bonds are formed in biological systems have remained undefined for the thirty years following the first report (2) of an organic phosphonate as a natural product. Synthetic sequences commonly used in the laboratory for construction of P-C bonds invariably require reduced forms of phosphates (viz. phosphonic and phosphinic acids) to serve as precursors in reactions with carbon electrophiles or nucleophiles. Some bacteria are able to utilize orthophosphite, which is an excellent reagent for P-C bond formation, as their sole source of phosphorus (3). Moreover, the bialaphos producing strains of Streptomycetes are known to synthesize a number of phosphonates which by virtue of their P-H bonds are potential precursors to P-C bond containing compounds (4). Thus, P-C bond formation in these organisms may occur via "conventional" chemical routes.

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Organisms which produce phosphonates but which do not metabolize reduced phosphates must necessarily form P-C bonds by reactions of phosphate esters, amines or anhydrides with carbon centered anions or their equivalents. One such organism, Tetrahymena pyriformis has been the subject of numerous investigations probing phosphonate synthesis and function. The results of these studies have suggested that the diacylglyceride phosphonoethylamines synthesized in this protozoan are produced for the purpose of constructing a lipase and phosphatase resistant membrane (5-6). The key phosphonate unit of these phosphonolipids is 2-aminoethylphosphonate (AEP), the most abundant and ubiquitous of all of the known phosphonate natural products. Because of the vital role AEP plays in this organism and its probable role as a precursor to a number of the naturally occuring phosphonates (4,7), knowledge about the AEP biosynthetic pathway is of fundamental importance.

Shortly after the discovery of AEP in T.pyriformis several laboratories set out to determine the structure of the organophosphate which serves as the substrate in the P-C bond forming process. Based on the radiolabeling patterns observed in AEP resulting from the reactions of $^{14}C$- or $^{32}P$-labelled metabolites in T.pyriformis whole cells or cellular homogenate, phosphoenol pyruvate (PEP) was shown to be a key precursor (8-12). Three different biosynthetic pathways leading from PEP to AEP were proposed. These are shown in Scheme 1. Pathways **a** (12) and **b** (10) lead directly to AEP while pathway **c** (11) leads directly to the AEP containing phosphonolipid. Horiguchi (12) showed that the synthesis of "free" AEP in T.pyriformis cellular homogenate occured at a significanlty faster rate than did the synthesis of lipid bound AEP and consequently dismissed pathway **c** from further consideration. Pathways **a** and **b** (which involve the rearrangement of PEP to phosphonopyruvate (P-pyr)) were distinguished on the basis of radioisotope dilution studies. Horiguchi (12) showed that added phosphonoalanine had no effect on the specific activity of [$^{14}C$]AEP produced from [$^{14}C$]PEP in T.pyriformis cellular homogenate, while added phosphonoacetaldehyde (P-ald) showed a significant effect. At a later date Horiguchi and Rosenberg (13) reported that they were able to trap the P-ald and P-pyr produced from PEP in the homogenate as the corresponding 2,4-dinitrophenylhydrazone derivatives. The structures of the hydrazones were, however, not rigorously demonstrated. This fact plus failure in our attempts to repeat the reported isolation of the P-ald and P-pyr hydrazone derivative prompted us to determine the validity of the proposed pathway, **a** by using a direct approach. Accordingly, the ability of the putative precursor PEP, and the intermediates, P-pyr and P-ald to support AEP biosynthesis in T.pyriformis cellular homogenate was tested.

## MATERIALS AND METHODS

General. Phosphonoacetaldehyde was prepared according to the method of LaNauze et. al. (14) and phosphonopyruvate was prepared according to the method of Anderson et al. (15). [$^{32}P$]Pi, [$\gamma$-$^{32}P$]ATP and [$^{14}C$]pyruvate were purchased from New England Nuclear. [$^{14}C$]PEP was prepared by reaction of ATP, $P_i$ and [$^{14}C$]pyruvate in the presence of pyruvate phosphate dikinase (isolated from Bacteriodes symbiosus (16)) and yeast inorganic pyrophosphatase (Sigma Chem. Co.). [$^{32}P$]PEP was prepared in the same manner by using [$\beta$-$^{32}P$]ATP and unlabelled pyruvate in place of unlabelled ATP and [$^{14}C$]pyruvate. The [$\beta$-$^{32}P$]ATP was generated from [$\gamma$-$^{32}P$]ATP by reaction with AMP in the presence of myokinase (Sigma Chem. Co.) followed by reaction with PEP in the presence of pyruvate kinase (Sigma Chem. Co.). $^{1}H$-nmr, $^{13}C$-nmr and $^{31}P$-nmr spectra were recorded with a Bruker AM-400 nmr (operating at 161 MHz for $^{31}P$). Silica

gel TLC analytical plates (Eastman Kodak Co.) were eluted with isopropanol:$H_2O$:$NH_4OH$ (7:5:1) solvent. The TLC chromatograms were developed by using $I_2$, ninhydrin spray or autoradiographic techniques.

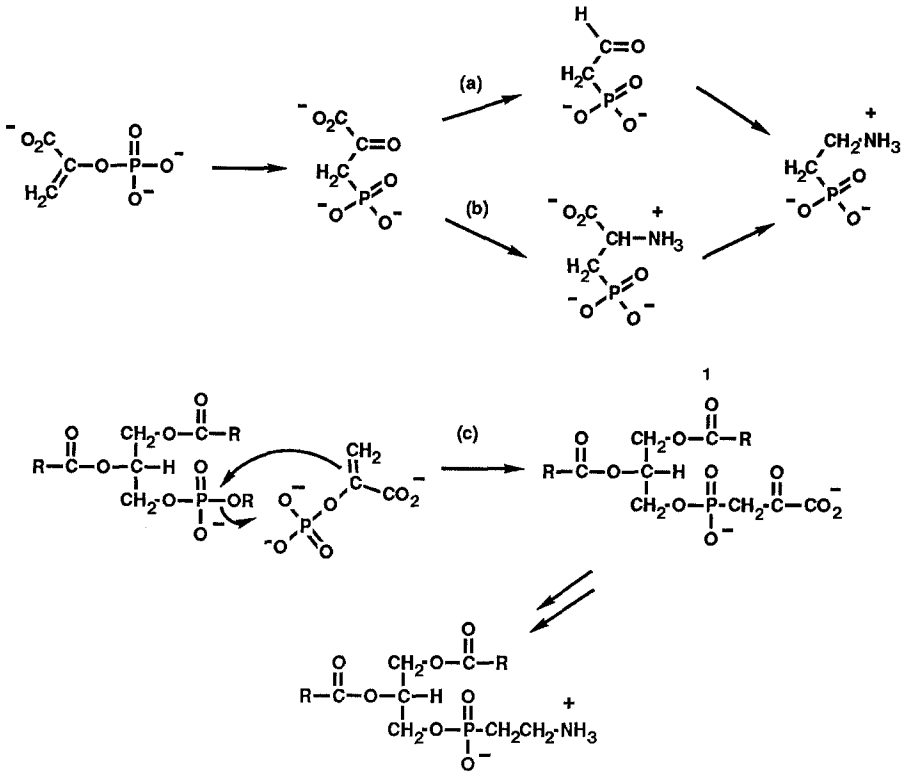
Cell growth, harvesting and lysis. The methods used for these tasks were adapted from the procedures reported by Horiguchi (12). Tetrahymena pyriformis W. was grown at 27°C on media consisting of Bacto-peptone (20g/l), yeast extract (5.0 g/l), glucose (2.0 g/l), NaCl (0.1 g/l), $CaCl_2$ (0.008 g/l) and KCl (0.004 g/l). Four day old stock cultures (5 ml) were used to innoculate 100 ml of media. The culture was agitated for 30 hr on a gyratory shaker after which time it was added to one liter of media. After an additional 20 hr of incubation a second liter of fresh media was added to the culture. After a 3 hr incubation period the culture was cooled to 4°C and the cells harvested by centrifugation. The average yield obtained was 2.6 ml of packed cells/liter of culture. The cells were washed with a solution containing 0.2 M sucrose and 0.18% NaCl and then homogenated at 0°C with a tissue homogenizer (Eberhart) fitted with a teflon pestle. One hundred ml of suspension buffer (50 mM glycine, 1 mM EDTA, 10 mM β-mercaptoethanol, 1.6% BSA, 1 mM 1,10 phenanthroline, 1 mM benzamidine hydrochloride hydrate, 50 μM phenylmethylsulfonyl fluoride and 50 μg/ml trypsin inhibitor) at pH 9.4 were used per 16 ml of packed cells (or the yield from 6 l of culture).

Reactions with T.pyriformis Cellular Homogenate. Reaction conditions and reaction fraction procedures were in part adapted from published procedures (12). Except where noted all reactions consisted of 20 ml of basal solution (50 mM $K^+$Hepes, pH 7.6, 10 mM $MgCl_2$, 0.4% BSA, 20 mM L-alanine, 2.5 mM pyridoxal phosphate and 2.5 mM thiamine pyrophosphate) and 20 ml of cellular homogenate. The reactants (PEP, phosphonopyruvate and phosphonoacetaldehyde) were added to this mixture to a concentration of 10 mM except where noted. Reaction mixtures were agitated on a gyratory shaker at 27°C for ca 12 hrs and terminated by addition of 40 ml of ice-cold 20% (w/v) trichloroacetic acid. The resulting mixtures were centrifuged at 1500g. Twice the precipitant was resuspended in cold 5% trichloroacetic acid and centrifuged. The trichloroacetic acid extracts were combined and extracted with diethylether. Two equivalent volumes of ethanol were added to the aqueous solution to precipitate glycogen. After centrification the supernatant was concentrated to dryness in vacuo. The residue was rehydrated in a small volume of water, and loaded on a 2.5 X 30 cm Dowex-50 ($H^+$) column. The column was eluted first with 300 ml of water and then with 300 ml of 3 M $NH_4OH$. The $H_2O$ fractions obtained from the reactions of unlabelled precursor were concentrated in vacuo, taken up in $D_2O$, adjusted to pH 8 with NaOH solution and analyzed for phosphonates and phosphates by $^{31}$P-nmr. The concentrated $H_2O$ fractions obtained from the reactions of the radiolabelled reactants were analyzed by using silica gel TLC. The $NH_4OH$ eluate was evaporated to dryness in vacuo and then rehydrated and applied to a 1.8 X 40 cm Dowex-1 ($CH_3COO^-$) column. The column was eluted with 200 ml water and then with 250 ml of 5% (v/v) acetic acid. The acetic acid fraction was concentrated to dryness in vacuo and analyzed by using P-31 nmr techniques or TLC techniques as described above. The AEP was purified from this fraction by using a 2.5 X 30 cm Dowex-50 ($H^+$) column and $H_2O$ as eluant. The AEP containing fractions were concentrated, taken up in $D_2O$, adjusted to pH 8 with NaOD and analyzed by using $^{31}$P-, $^{13}$C- and $^{1}$H-nmr techniques.

## RESULTS AND DISCUSSION

In this study the putative AEP precursors, PEP, P-pyr and P-ald were incubated with buffered T.pyriformis homogenate to which $Mg^{2+}$, alanine/pyridoxal-P and in most instances, thiamine pyrophosphate had been added. After specified incubation periods the reaction mixtures were fractionated by using the series of precipitation and column chromatographic steps described by Horiguchi (12). The structures of the reaction products were identified by using silica gel TLC techniques and $^{31}$P-, $^{1}$H- and $^{13}$C-nmr techniques to compare their chromatographic and spectral properties to those of known compounds.

First, the fate of PEP in cellular homogenates containing $Mg^{2+}$ and alanine/pyridoxal-P was examined. [$^{32}$P]PEP and [$^{14}$C]PEP were reacted independently and the products formed from these radiolabelled substrates were examined at varying conversion by TLC-chromatographic analysis of aliquots taken from the reaction mixtures. Within 2 hrs ca 50% of the PEP had been



Scheme 1

consumed. Audioradiographic analysis of the TLC plates revealed that the two radiolabelled products of the [$^{32}$P]PEP reaction chromatographed with the same $R_f$ values as $P_i$ and phosphoglycerate. The radiolabelled products of the [$^{14}$C]PEP reaction were phosphoglycerate and pyruvate. The catalyzed hydrolysis and hydration reactions of PEP appeared to occur at comparable rates . A second set of [$^{32}$P]PEP and [$^{14}$C]PEP reactions run for 12 h were fractionated for AEP and phosphonoalanine. The Dowex-1 acetic acid eluate or "AEP fraction" contained AEP in 2% yield in the case of the [$^{32}$P]PEP reaction and 5% in case of the [$^{14}$C]PEP reaction. No phosphoalanine formation in either reaction mixture was observed.

Because we expected that the second step of pathway a (Scheme 1), would be catalyzed by an α-ketodecarboxylase, we tested the effect of including the α-ketodecarboxylase cofactor, thiamine pyrophosphate in the cellular homogenate. The yield of AEP from PEP increased approximately ten fold. The amount of AEP derived from the PEP well exceeded the amount of endogenous AEP obtained by fractionation of the cellular homogenate - cofactor mixture of the control reaction. Thus, we were able to examine AEP formation from unlabelled of PEP as well as the other two suspected precursors by using $^{31}$P-nmr techniques for quantitation. On four separate occasions PEP, P-pyr and P-ald were incubated for 12 hr with fresh cofactor fortified T.pyriformis homogenate. Control reactions were carried out without precursor or with boiled rather than fresh homogenate and analyzed along with of the test reactions. The results of these experiments shown

Table I. Reaction of AEP Precursors with Tetrahymena Cellular Homogenate in the Presence of Alanine, Pyridoxal Phosphate, Thiamine Pyrophosphate and $MgCl_2$

| Reactant | AEP Isolated [μmol]/Percent Conversion | | | |
|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Trial 4 |
| None[a] | 4 μmol | 6 μmol | 5 μmol | 0 μmol |
| PEP [200 μmol] | 12 μmol/6% | 25 μmol/13% | 75 μmol/37% | 36 μmol/18% |
| P-pyr [130-200 μmol][b] | 11 μmol/8% | 10 μmol/11% | 10 μmol/8% | 22 μmol/16% |
| P-ald [200 μmol] | 19 μmol/9% | 133 μmol/66% | 166 μmol/83% | 80 μmol/40% |

[a]These are control reactions to which the addition of the PEP, P-pyr and P-Ald had been ommitted.

[b]The trial 1 reaction mixture contained 130 μmol, trial 2 and trial 3 reaction mixtures contained 136 μmol and the trial 4 reaction mixture contained 200 μmol.

See Materials and Methods for further detail.

in Table I, serve as the first direct evidence for the precursor roles of P-pyr and P-ald in AEP biosynthesis. The AEP formed from each of the precursors was further purified by performing a second Dowex-50 column chromatography. The $^{1}H$-nmr, $^{31}P$-nmr and $^{13}C$-nmr spectra of material obtained in this manner were identical to those measured for authentic AEP.

As indicated by the data shown in Table I, AEP yields varied from one experiment to the next. However, we found that the yields obtained from duplicate reactions in which the same cellular homogenate was used were reproducible. Thus, it appears that differences in the cell batches used and/or the manner in which cell lysis is achieved may be the cause of the variation observed in the AEP yields.

The data shown in Table I indicate that the conversion of P-pyr to AEP is generally less efficient than is the conversion of PEP to AEP. This result may suggest that the P-pyr formed from reaction of PEP in the phosphomutase active site is not released to the surrounding media but is instead "shuttled" into the active site of a neighboring P-pyr decarboxylase. Guided diffusion of the product from one enzyme active site into the active site of a second enzyme for which it serves as substrate has been previously described (17). If alternate reaction pathways for P-pyr consumption exist and these pathways are more efficient than alternate pathways leading to PEP consumption then the yield of AEP from PEP is expected to exceed that from P-pyr. We pursued this idea by examining the lifetimes of P-pyr and PEP in the cellular homogenate and the reaction products generated from these substances. Specifically, the P-pyr reaction was assayed for unreacted P-pyr as a function of incubation time. We found that within a reaction period of 15 min the P-pyr had been completely consumed. At 2 min, 35% of the original P-pyr remained but the other 65% had been converted to AEP (~9%), PEP (~11%) and $P_i$ plus pyruvate (~45%). In contrast, PEP incubated with the cellular homogenate for 2 min under the exact same conditions was recovered in greater than 90% yield.

The observed formation of PEP from P-pyr in the T.pyriformis cellular homogenate represents the first observation suggesting that the conversion of PEP to P-pyr may not be an energetically favorable process. In fact, a reaction carried-out with T.pyriformis cellular

homogenate and P-pyr in the presence of $Mg^{2+}$ but in the absence of the alanine/pyridoxal phosphate and thiamine pyrophosphate produced PEP in 40% yield. PEP was not found in the control reaction in which 10 mM pyruvate and $P_i$ were substituted for the P-pyr. Furthermore, the observation that the homogenate catalyzes the formation of $P_i$ and pyruvate from P-pyr at a rate that exceeds the rate at which it catalyzes the hydrolysis of PEP to these products, indicates that the T.pyriformis homogenate contains a phosphonatase which catalyzes the hydrolysis of P-pyr. Either an unfavorable equilibrium (viz. PEP P-pyr) or an efficient reaction pathway leading to rapid P-pyr consumption (viz. P-pyr $\Rightarrow\Rightarrow$AEP or P-pyr $\Rightarrow$ $P_i$ + pyruvate) could account for the failure of several laboratories (including our own) to observe direct formation of P-pyr from PEP in T.pyriformis cellular homogenate.

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