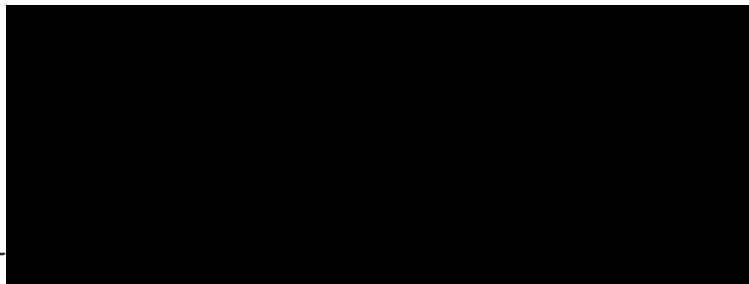STUDIES ON 2-AMINOETHYLPHOSPHONIC
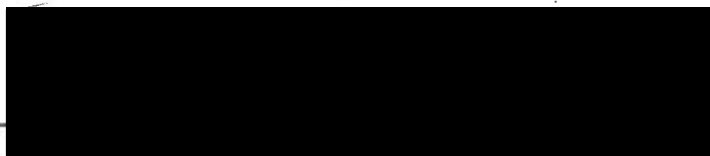
ACID IN HUMAN TISSUES

by

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A Thesis
Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
June 1972

APPROVED:

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(Professor in Charge of Thesis)

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(Chairman, Graduate Council)

AN ABSTRACT OF THE THESIS OF

Jack Abraham Alhadeff for the   Ph.D.   in   Biochemistry
         (Name)   (Degree)   (Major Department)

Date of receiving this degree: June , 1972

Title: Studies on 2-Aminoethylphosphonic Acid in Human Tissues

Approved: _____
                 (Professor in Charge of Thesis)

Up to the present time a specific assay method has not been
available for detection of 2-aminoethylphosphonic acid (AEP), an unusual
naturally occurring β-amino acid with the unique covalent carbon-
phosphorus bond. A prerequisite for an investigation of the occurrence
of AEP in human tissues is the availability of a sensitive and specific
method for its determination. Such a method has been developed for the
detection of AEP in biological materials. This new assay involves
acetylation and methylation of hydrolysates of tissues or tissue
extracts, detection by automated amino acid analysis and gas-liquid
chromatography, isolation by preparative gas-liquid chromatography, and

unambiguous characterization of the volatile AEP derivative (dimethyl 2-acetamidoethylphosphonate) by mass spectrometry.

The new assay has been used to study the occurrence, distribution and concentration of AEP in human tissues. Several different tissues with diverse compositions and functions were investigated with the hope of better localizing the occurrence of AEP and thus gaining some insight into its possible functions. However, the results indicate a ubiquitous occurrence and distribution of AEP in human tissues. AEP was found in human brain (0.1 mg AEP/g wet tissue), liver (0.2 mg AEP/g wet tissue), skeletal muscle (0.4 mg AEP/g wet tissue), and heart (0.6 mg AEP/g wet tissue). Brain and liver were fractionated into hexane soluble (nonpolar lipids), methanol soluble (polar lipids) and hexane-methanol insoluble (Proteinaceous residue) fractions and the concentration of AEP in these fractions was determined. The distribution between fractions differed considerably but differently in the two tissues. However, for both tissues the protein-rich extracts contained the highest concentration of AEP.

Because AEP is thought by some to be a component of membrane phospholipids, human red blood cell membranes (ghosts) were isolated and investigated for the presence of AEP. AEP was found to be present at a level of 0.1 mg AEP per gram of red blood cell ghosts (wet weight).

Biosynthetic studies were carried out to determine whether a mammal is capable of synthesizing AEP. Both *in vitro* (rat liver

slices) and *in vivo* (rat) experiments were done using several radio-active compounds (U-^{14}C-glucose, ^{14}C$_3$-pyruvate and ^{32}P-orthophosphate) which are known to be precursors in the biosynthesis of AEP by *Tetra-hymena pyriformis*. In no case was there an incorporation of label greater than 0.001%.

Dedicated to

G. Doyle Daves, Jr.,

a constant source of encouragement and inspiration
during the fulfillment of this work.

Acknowledgements

I wish to express my thanks to the faculty, staff and students of the Department of Biochemistry for their patience, encouragement and help during the preparation of this work.

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I am particularly indebted to Professors John T. Van Bruggen for generously providing the facilities of his laboratory for a part of this investigation and Richard T. Jones for acting as my advisor at the Medical School.

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ABBREVIATIONS

AEP	Aminoethylphosphonic Acid (Ciliatine)
TCA	Trichloroacetic Acid
PA	Phosphonoalanine
PEP	Phosphoenolpyruvate
Pi	inorganic phosphate
NMR	Nuclear Magnetic Resonance (Spectrometry)
TMS	tetramethylsilane
tlc	thin layer chromatography
Glc	gas-liquid chromatography
IR	infrared
m/e	mass/charge
rbc	red blood cell

Chapter I - INTRODUCTION

In 1959 Horiguchi and Kandatsu discovered 2-aminoethylphosphonic acid (AEP) in ciliated protozoa of sheep rumen and designated this unique new compound ciliatine (1,2). This discovery of a β-amino acid with a covalent carbon-phosphorus bond in nature led to the emergence of a potentially important new field of biochemistry. In this brief introduction only those topics which are relevant for understanding later sections of the thesis will be reviewed. More general reviews are available (3,4). Figure 1 gives the structure of AEP as well as the structures of several other phosphonic acids which will be discussed.

A. Occurrence and Distribution of AEP

After its discovery and isolation, most work related to AEP was concerned with its occurrence in microorganisms and marine life (3,4). Subsequently, four other naturally occurring phosphonic acids were found (3-7), three of which were simple N-methyl derivatives of AEP, the fourth being α-amino-β-phosphonopropionic acid (or phosphonoalanine, see Figure 1). AEP and other biological phosphonates have been found in many sources including *Tetrahymena pyriformis* (5,8-10),
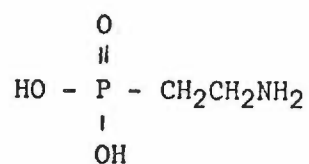
$$
\begin{array}{l}
\quad\ \ \overset{\displaystyle O}{\overset{\displaystyle \|}{\ }} \\
HO - P - CH_2CH_2NH_2 \\
\quad\ \ | \\
\quad\ \ OH
\end{array}
$$
Aminoethylphosphonic Acid (AEP)
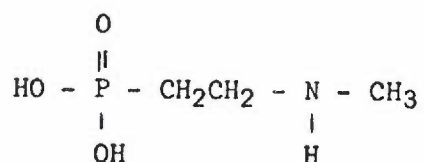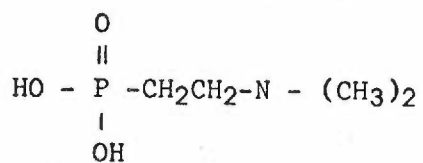
$$
\begin{array}{l}
\quad\ \ \overset{\displaystyle O}{\overset{\displaystyle \|}{\ }} \\
HO - P - CH_2CH_2 - N - CH_3 \\
\quad\ \ | \qquad\qquad\ | \\
\quad\ \ OH \qquad\quad\ \ H
\end{array}
$$
N-methyl Aminoethylphosphonic Acid

$$
\begin{array}{l}
\quad\ \ \overset{\displaystyle O}{\overset{\displaystyle \|}{\ }} \\
HO - P - CH_2CH_2 - N - (CH_3)_2 \\
\quad\ \ | \\
\quad\ \ OH
\end{array}
$$
N,N dimethyl Aminoethylphosphonic Acid

$$
\begin{array}{l}
\quad\ \ \overset{\displaystyle O}{\overset{\displaystyle \|}{\ }} \\
-O - P - CH_2CH_2 - \overset{+}{N} - (CH_3)_3 \\
\quad\ \ | \\
\quad\ \ OH
\end{array}
$$
N,N,N trimethyl Aminoethylphosphonic Acid (Choline Phosphonate)
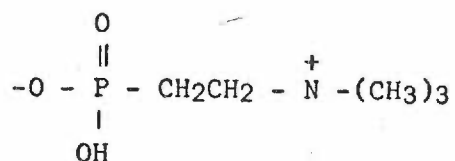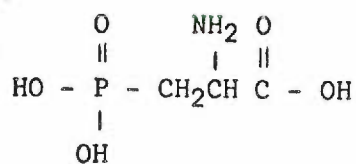
$$
\begin{array}{l}
\quad\ \ \overset{\displaystyle O}{\overset{\displaystyle \|}{\ }} \quad\ \overset{\displaystyle NH_2}{\overset{\displaystyle |}{\ }} \ \overset{\displaystyle O}{\overset{\displaystyle \|}{\ }} \\
HO - P - CH_2CH \ C - OH \\
\quad\ \ | \\
\quad\ \ OH
\end{array}
$$
α-amino-β-phosphonopropionic Acid (Phosphonoalanine; PA)

Figure 1 - Structures of Several Naturally Occurring Phosphonic Acids

sea anemone (6,7,11-14), marine invertebrates in the phyla Coelen-
terata, Mollusca and Echinodermata (15), abalone (16), mussel (17,18),
the salt water crab (19), various aquatic animals (20-24), garden
snail and garden slug (25) and mycobacteria (26). This list is not
a complete documentation but indicates the prevalence of these
covalently-bonded carbon-phosphorus compounds in many diverse living
forms.

Little work has been done on phosphonic acids in mammalian
systems. AEP has been isolated from goat liver (27) and beef brain
(28) and Alam and Bishop have claimed the presence of trimethyl-
aminoethylphosphonic acid (choline phosphonate) in human aortae (29).
Data in this thesis, portions of which have been published (30,31),
document the presence of AEP in mammalian tissues: human brain,
liver, skeletal muscle, heart, and blood. A very recent paper (32)
indicates that another laboratory has also found AEP in several human
tissues: brain, liver, heart, kidney, intestine, spleen, adrenal and
aorta. These recent findings, together with our own, suggest that AEP
may be an important compound in human biochemistry.

B. Tissue Concentration of AEP

The concentration of AEP in the organisms in which it has been
found ranges from micrograms to milligrams per gram of tissue. Shimizu
et al. found only small amounts in beef brain: 2 µg AEP per gram of
wet tissue (28). DeKoning isolated approximately 21 µg AEP per gram

of abalone (16) and Horiguchi and Kandatsu isolated about 0.3 mg AEP
per gram of ciliated protozoa (1,2). Quin has claimed that as much
as one percent of the dry weight of sea anemone is AEP (15). Human
tissues appear to be relatively rich in AEP containing from 0.1 mg to
0.6 mg of AEP per gram of wet tissue (30,31). The details of the
occurrence, distribution and concentration of AEP in human tissues
will be presented in a later section.

C. Naturally Occurring Molecular Forms of AEP

AEP exists both in free and in covalently bound forms in tissues.
Both the snail and slug contain AEP in the free form as well as in
lipid-bound form (25). Furthermore, both of these organisms have AEP
in the insoluble residue after extraction of lipids. This residue
would contain the proteins and therefore AEP could be protein-bound.
Quin has shown that a considerable portion of the AEP in the sea
anemone *Metridium dianthus* resides in the "proteinaceous residue"
(14). Analysis of this "proteinaceous residue" (ethanol-chloroform
insoluble fraction) showed that 1.1 percent of it was AEP (14). In
a later paper (15) Quin further purified the proteinaceous residue by
solubilizing it in 1 M sodium hydroxide for 30 minutes and then pre-
cipitating the protein with 30% trichloroacetic acid (TCA). He found
AEP in this TCA precipitate and in a fraction further purified on
Sephadex G-25 after hydrolysis with pepsin (15). A methyl derivative
of AEP, 2-(methylamino)ethylphosphonic acid, has also been isolated

from the proteinaceous residue of the sea anemone, *Anthopleura xanthogrammica* (7). Stevenson has claimed that AEP is a constituent of two proteases (Proteases A and B) which he has isolated from the sea anemone, *Metridium senile* (33). Protease A contained six residues of AEP while Protease B contained four residues as determined by the automatic amino acid analyzer (33).

Quin has speculated on the ways in which AEP might be bonded in proteins (15). Figure 2 illustrates his concept of AEP bonding which may occur through one or both acidic functions of the phosphonic acid group (A), through the amino group (B), or through both amino and phosphonic acid groups (C). Quin decided against the first possibility (A) through dinitrophenylation studies and favors possibilities B and C (15).

The majority of AEP in the various organisms in which it is found occurs covalently bound to tissue phospholipids. For example, ceramide aminoethylphosphonate (CAEP)* has been shown to be present in sea anemone (12,13), rumen protozoa (34), bivalves, snails and cephalopods (20) and various shellfish (18,23). Simple N-acyl and N-methyl derivatives of CAEP (acylated or methylated on the free amino group of CAEP) have been isolated from the mussel, *Corbiculae sandai* (17), from the viscera of the marine shellfish, *Turbo cornutus* (35) and from the marine snail, *Monodonta labio* (21). The acid residue in these compounds

* See Figure 3 for Structure

$$
\begin{array}{l}
\quad\quad\quad\quad\quad\quad O \\
\quad\quad\quad\quad\quad\quad \| \\
A \quad\quad\quad -CH_2-X-P\ -CH_2CH_2NH_2 \\
\quad\quad\quad\quad\quad\quad\ | \\
\quad\quad\quad\quad\quad\quad\ OH
\end{array}
$$

$$(X = NH\ or\ O)$$

$$
\begin{array}{l}
\quad\quad\quad O \quad\quad\quad O \\
\quad\quad\quad \| \quad\quad\quad\ \| \\
B \quad\quad -C-NHCH_2CH_2P\ -OH \\
\quad\quad\quad\quad\quad\quad\quad\ | \\
\quad\quad\quad\quad\quad\quad\quad\ OH
\end{array}
$$

$$
\begin{array}{l}
\quad\quad\quad O \quad\quad\quad\quad O \quad\ R \quad O \\
\quad\quad\quad \| \quad\quad\quad\quad \| \quad\ | \quad\ \| \\
C \quad\quad -C-NHCH_2CH_2-P\ -X-CH\ -C-NH- \\
\quad\quad\quad\quad\quad\quad\quad\quad\ | \\
\quad\quad\quad\quad\quad\quad\quad\quad\ OH
\end{array}
$$
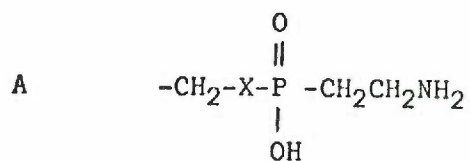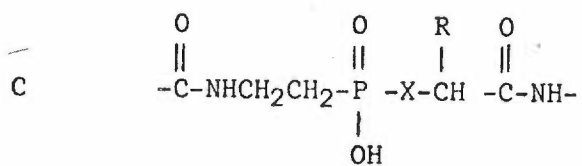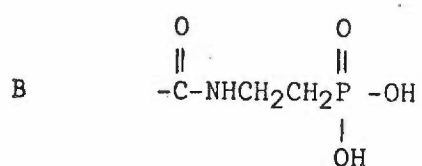
$$(X = NH\ or\ O)$$

Figure 2 - Possible Methods of AEP Bonding in Proteins as suggested
by Quin (15).
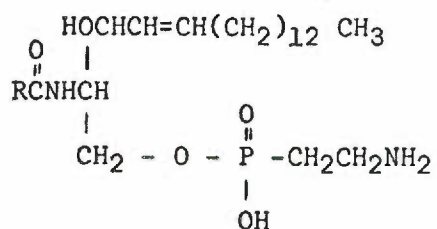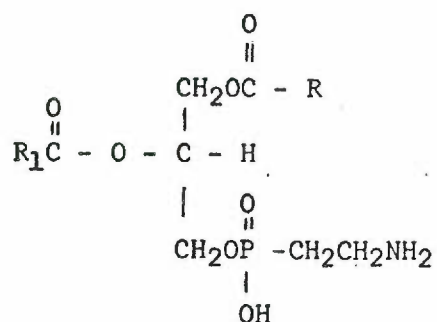
$$
\begin{array}{l}
\text{HOCHCH=CH(CH}_2)_{12}\text{ CH}_3 \\
\quad\quad\underset{\overset{\parallel}{O}}{\text{R}}\overset{|}{\text{C}}\text{NHCH} \\
\quad\quad\quad\quad | \quad\quad\quad\overset{O}{\overset{\parallel}{} } \\
\quad\quad\quad\text{CH}_2 - \text{O} - \overset{\parallel}{\text{P}} -\text{CH}_2\text{CH}_2\text{NH}_2 \\
\quad\quad\quad\quad\quad\quad\quad | \\
\quad\quad\quad\quad\quad\quad\quad\text{OH}
\end{array}
$$

Ceramide Aminoethylphosphonate
(CAEP)

$$
\begin{array}{l}
\quad\quad\quad\quad\overset{O}{\overset{\parallel}{}} \\
\quad\quad\quad\text{CH}_2\text{OC} - \text{R} \\
\quad\overset{O}{\overset{\parallel}{}}\quad\quad | \\
\text{R}_1\text{C} - \text{O} - \text{C} - \text{H} \\
\quad\quad\quad\quad | \quad\quad\overset{O}{\overset{\parallel}{}} \\
\quad\quad\quad\text{CH}_2\text{OP} -\text{CH}_2\text{CH}_2\text{NH}_2 \\
\quad\quad\quad\quad\quad | \\
\quad\quad\quad\quad\quad\text{OH}
\end{array}
$$

Phosphatidylaminoethylphosphonate

$$
\begin{array}{l}
\quad\quad\quad\quad\text{CH}_2 - \text{O-CH=CH} - \text{R} \\
\quad\overset{O}{\overset{\parallel}{}}\quad\quad | \\
\text{R}_1\text{C} - \text{O} - \text{CH} \\
\quad\quad\quad\quad | \quad\quad\overset{O}{\overset{\parallel}{}} \\
\quad\quad\quad\text{CH}_2 - \text{OP} - \text{CH}_2\text{CH}_2\text{NH}_2 \\
\quad\quad\quad\quad\quad | \\
\quad\quad\quad\quad\quad\text{OH}
\end{array}
$$

Plasmalogen Ciliatine

$$
\begin{array}{l}
\text{CH}_2\text{OH} \\
\quad | \\
\text{HO-CH} \\
\quad | \quad\quad\overset{O}{\overset{\parallel}{}} \\
\text{CH}_2\text{OP} - \text{CH}_2 - \text{CH}_2\text{NH}_2 \\
\quad\quad | \\
\quad\quad\text{OH}
\end{array}
$$
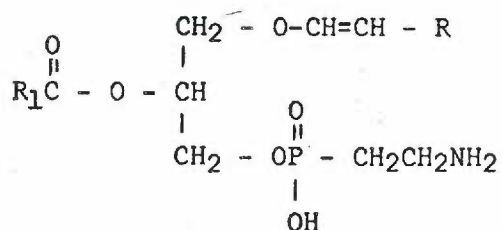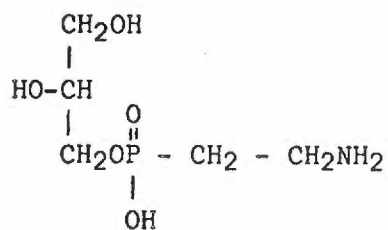
Glycerylaminoethylphosphonate

Figure 3 - Naturally Occurring Phospholipid Forms of AEP

has usually been palmitic or stearic acid.

Phosphatidylaminoethylphosphonate[*], the phosphonic acid analogue of phosphatidylaminoethylphosphate (cephalin), has also been isolated. This compound is present in rumen protozoa (34), *Tetrahymena pyriformis* (8,9) and possibly in mycobacteria (26). Rumen protozoa also contain a similar compound (plasmalogen ciliatine[*]) which contains an ether linkage as well as the carboxylic acid ester linkage (8,34). This compound is probably also present in membrane lipids of *Tetrahymena pyriformis* (36).

Glycerylaminoethylphosphonate[*] is still another molecule in which AEP is bound. This compound has been found in ciliary phospholipids from *Tetrahymena pyriformis* (37) and in the sea anemone, *Anthopleura elegantissima* (11).

D. Biosynthesis of AEP

Several papers have been concerned with the biosynthesis of AEP. That *Tetrahymena pyriformis* can biosynthesize AEP has been adequately demonstrated by several laboratories (38-42). Warren (38) has demonstrated that the carbon skeleton of AEP can come from $^{14}C_1$ - glucose, $^{14}C_2$ - glucose, $^{14}C_6$ - glucose, $^{14}C_3$ - pyruvate, $^{14}C_2$ - acetate and L - $^{14}C_3$ - alanine. Furthermore, $^{14}C_1$ - glucose, $^{14}C_2$ - glucose,

_____

[*] See Figure 3 for Structure

$^{14}C_3$ - glucose and $^{14}C_4$ - glucose were incorporated into phosphono-alanine (PA) (See Figure 1) with nearly the same specific activity as into AEP, suggesting a common biosynthetic pathway. ^{32}P - phosphoenol-pyruvate (PEP) served as a precursor for both PA and AEP. With regard to his experimental findings, Warren proposed the following plausible biosynthetic pathway for AEP (involving the intramolecular rearrangement of PEP):

(3-phosphonopyruvic acid)

(PEP)

(PA)   (AEP)
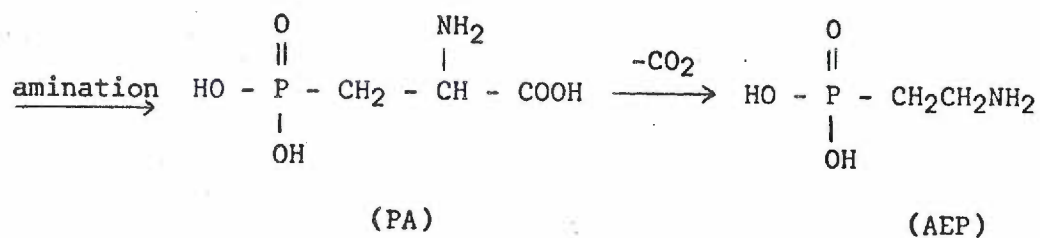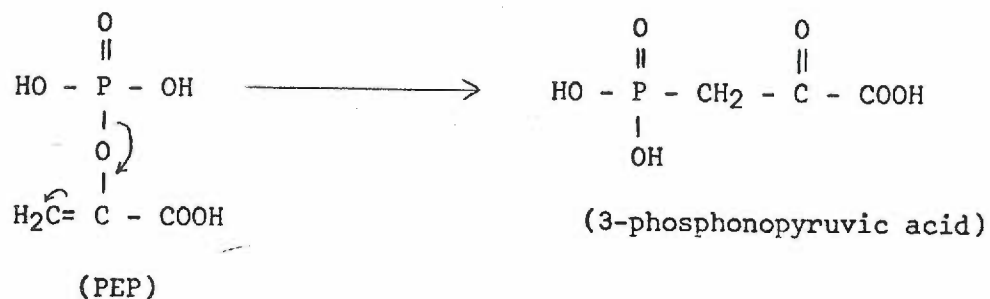
Liang and Rosenberg (40) also found that glucose, pyruvate or acetate could serve as the precursor for the carbon skeleton of AEP. Furthermore, they showed that $^{32}PO_4$ is incorporated *in vivo* into AEP and PA. Horiguchi, Kittredge and Roberts (41) confirmed that

PEP could serve as a precursor to AEP and gave evidence that AEP was labelled by $^{14}C_2$ and/or $^{14}C_3$, but not by the carboxylic carbon ($^{14}C_1$), of succinate, fumarate and malate. Segal has proposed a theoretical biosynthetic pathway from ethanolamine to 2-AEP (43). However, his pathway is not experimentally verifiable in that ^{14}C - ethanolamine is not incorporated into AEP (36,40). In summary, it seems well established that several glycolytic and tricarboxylic acid cycle intermediates can serve as precursors for the biosynthesis of AEP in Tetrahymena.

E. Putative Functions of AEP

To date, no function for AEP has been proven, although several speculations as to its function have been offered. Kennedy and Thompson have suggested that AEP may be involved as a structural component in membrane phospholipids (44). In support of this suggestion, they have shown that 60% of the phospholipids from the membrane sheath of *Tetrahymena pyriformis* cilia contain AEP. They contend that the presence of AEP in phospholipids might prevent their degradation by phospholipases in the external environment. They substantiated this contention by demonstrating that the surface membrane fractions of Tetrahymena, which would be exposed to the external environment, contain a higher percentage of phosphonolipids than do the intracellular membranes. Earlier, Thompson had shown that phosphatidylaminoethyl-phosphonate was probably a component of membranes in Tetrahymena (36).

Furthermore, Rosenberg (45) and Chou and Scherbaum (46) have also given
evidence that AEP might be involved as a structural material of cell
membranes.

A role in phosphate metabolism has also been considered for AEP
(47-53). Rosenberg and La Nauze (47,48) have shown that *Bacillus
cereus* can utilize AEP as a sole source of phosphorus and that phospho-
noacetaldehyde is a metabolite of AEP in its catabolism to inorganic
phosphate (49). They have recently purified an enzyme in *B. cereus*
(2-phosphonoacetaldehyde phosphonohydrolase) which catalyzes the
cleavage of the carbon-phosphorus bond of 2-phosphonoacetaldehyde (50).
Figure 4 depicts this catabolism of AEP, at least as it occurs in
B. cereus. Zeleznick et al. (51) have demonstrated that *E. coli* can
also utilize the phosphorus of AEP and Harkness has shown (52) that
nine out of ten bacterial strains tested were able to utilize the phos-
phorus of aminoalkylphosphonic acids. In a previous communication (30)
we speculated that since the catabolite of AEP, phosphonoacetaldehyde,
is isoelectronic with known biological metaphosphate - transfer agents
(e.g., phosphoenolpyruvate), AEP may be involved in high-energy
phosphate metabolism. Roberts has shown that AEP can be transaminated
in Tetrahymena and sea anemone homogenates with α-keto-glutarate to
phosphonoacetaldehyde (53). The carbon-phosphorus bond of phosphono-
acetaldehyde is labile, thus providing a mechanism for making available
the inorganic phosphate and the carbon skeleton of AEP.

Finally, a structural role for AEP in proteins has been postulated
(7,14,15). The data which lend support to this possible function have

$$
\begin{array}{c}
O \\
\parallel \\
H_2N\text{-}CH_2\text{-}CH_2\text{-}P\text{-}OH \\
| \\
OH
\end{array}
$$

Pyruvate
alanine ⤴

oxidative deamination

Pyridoxal
phosphate ↓

$$
\begin{array}{c}
O \quad\ O \\
\parallel \quad \parallel \\
HC\text{-}CH_2\text{-}P\text{-}OH \\
| \\
OH
\end{array}
$$
(2-Phosphonoacetaldehyde)

2-phosphonoacetaldehyde
phosphonohydrolase

↓

$$
\begin{array}{c}
O \\
\parallel \\
P_i + CH_3CH
\end{array}
$$
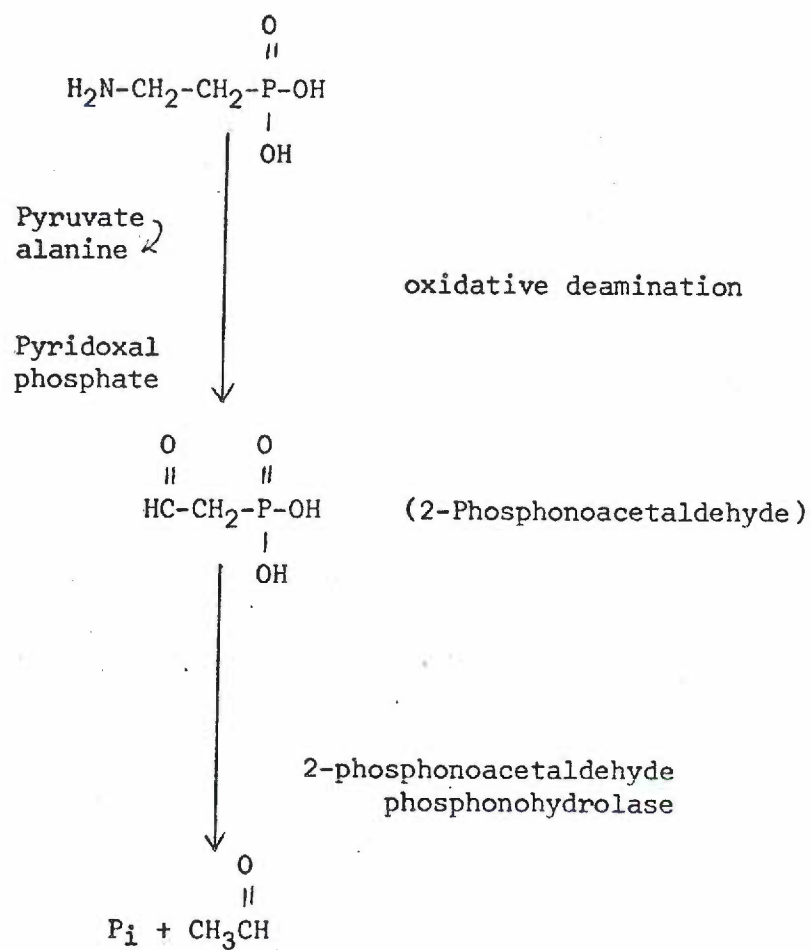
Figure 4 - Catabolism of AEP by *Bacillus cereus* (49).

already been discussed in the section on the "naturally occurring molecular forms of AEP." Although the data are not compelling as evidence for a covalent linkage of AEP to proteins, they suggest that AEP is at least associated with protein, perhaps in some nonspecific way.

F. Previous Methodology

Many workers in the field of phosphonic acids have relied upon Quin's indirect method for detection of AEP (15). However, Quin's method is a general one which assesses the amount of phosphorus covalently bound to carbon rather than assaying directly for a particular phosphonic acid like AEP. His assay involves determining total phosphorus and hydrolyzable phosphorus and ascribing the difference between these two values to phosphonate phosphorus. Quin determined total phosphorus using the Schoniger oxygen flask method (54) and determined phosphate phosphorus by hydrolysis with 6N HCl for 48 hours at 105 ± 2°C (15). Quin's method, and slight modifications of it (11,44,55,56), have been successfully used to locate phosphonic acids in various tissues or tissue extracts. However, isolation by standard chromatographic and electrophoretic techniques has been necessary to determine which phosphonic acids were present in the various organisms and tissues investigated. Quin realized the limitations of his indirect methodology and predicted that nuclear magnetic resonance (NMR) spectrometry might be of use for direct

detection of the C-P bond (15).

Recently two laboratories have successfully applied NMR techniques for detecting phosphonic acids (57,58). Baer and his group (57) have been able to use proton NMR to detect C-P compounds because the hydrogens of a methylene group in a phosphate (P-O-CH$_2$-) and the corresponding ones in a phosphonate (P-CH$_2$-) give rise to signals in very different regions of the spectrum. The magnetic anisotropy of oxygen deshields the methylene protons of the former and hence the signal from the methylene protons of the latter (the phosphonate) appear at higher magnetic fields relative to tetramethylsilane (TMS). This NMR technique has been used to differentiate glycerylaminoethylphosphate from glycerylaminoethylphosphonate and may be useful for studies differentiating other phospholipids from phosphonolipids in natural source materials.

Glonek and coworkers have used ^{31}P NMR for the determination of biological phosphonates (58). Their method relies on the fact that the chemical shift of phosphonate-phosphorus is considerably different from that of other forms of naturally-occurring phosphorus. Using this method they were able to demonstrate the presence of phosphonates in chloroform-soluble solutions of lipid extracts of various organisms. However, their method is not very sensitive and can only detect phosphonate-phosphorus at a total phosphorus concentration of 10^{-3} to 10^{-4} M (58).

Several other standard biochemical methods have been applied for the detection and isolation of AEP. Since AEP is an amino acid and

reacts with ninhydrin (59), the automatic amino acid analyzer has been used for detecting AEP in tissue hydrolysates (5,9,20,30,46). AEP is very acidic, elutes near the front and is readily separated from its phosphate analogue, phosphorylethanolamine (19,20). This detection of AEP by the amino acid analyzer relies on the fact that AEP is separated from the other constituents of tissue hydrolysates (e.g., amino acids) by ion-exchange chromatography. In fact ion-exchange chromatography using the strongly acidic cation exchanger Dowex 50(H^+ form) and/or the weakly basic anion exchanger Dowex 1 (acetate form) has been employed in most isolation schemes for AEP (1,2,14-16,22,26,27,60). Ion-exchange chromatography is also useful in separating orthophosphate from AEP (45) and, as already mentioned, in separating AEP from phosphorylethanolamine (61).

Thin layer chromatography (tlc) on various supports including layers of cation exchangers (62), paper chromatography (6,11,25,30,36, 46,60) and electrophoresis (5,11,25,48,60) have also been used to detect AEP. However, none of these chromatographic techniques is highly sensitive and specific, and they can yield false positives, particularly when used to study complex tissue (or tissue extract) hydrolysates.

After the new assay for AEP (described in Chapter III) had been developed, it was discovered that Karlsson had reported a similar assay using gas liquid chromatography and mass spectrometry of a trimethylsilyl (TMS) derivative of AEP (62).

G. Statement of the Problem and Goals of the Research

 The preceding brief review serves to introduce the subject matter
and the three research problems of this thesis. It was apparent that
a precise and sensitive detection method for AEP was lacking. There-
fore, the development of a new, highly sensitive and unambiguous assay
technique for the detection of AEP in biological materials was a
primary goal of the research since such new methodology was necessary
for undertaking studies to supplement the meager data on the occurrence,
distribution and concentration of AEP in mammalian systems. Several
tissues with diverse compositions and functions were investigated with
the intent of better localizing the occurrence of AEP. It was thought
that such studies might indirectly provide insight into possible
function(s) of AEP.

 Once the methodology was developed and the apparent ubiquitous
occurrence of AEP in human tissues was documented, the question as to
its origin in mammalian tissues was considered. At least three
possibilities presented themselves: AEP could be 1) ingested in the
diet, 2) biosynthesized by the symbiotic microorganisms inhabiting
mammals, or 3) biosynthesized by the mammalian organisms themselves.
The third portion of this research thesis is concerned with this last
possibility; that is, the question of whether mammals are capable of
biosynthesizing AEP. Demonstration of AEP biosynthesis in a mammal
would establish the importance of this compound in mammalian biochemistry.

 The organization of the remainder of this thesis will be as follows:

First, the materials and methods used in the research will be detailed.

Secondly, the experimental and results section will be presented in three chapters:

      1. Development of a new assay technique for the detection of AEP.

      2. Studies on the occurrence, distribution and concentration of AEP in human tissues.

      3. Studies on the biosynthesis of AEP in a mammalian system.

Thirdly, the experimental results will be discussed and interpreted with respect to the results of other investigators and possible implications of the findings will be discussed.

Finally, a brief summary of the results and conclusions will be presented.

Chapter II - <u>MATERIALS AND METHODS</u>

A. <u>Reagents and solvents</u> were of Analytical Reagent (A.R.) quality.
    The hexane used for tissue extraction was redistilled.

    1. <u>AEP</u> was synthesized according to the method of Kosolapoff (64)
        or purchased from Calbiochem (Los Angeles, California).

    2. <u>Amino acids</u> (Glu, Val, Phe, Thr, Asp, β-Ala) were purchased
        from Schwarz Bioresearch, Inc. (Orangeburg, New York).

    3. D-Glucose - $^{14}C(U)$ (4.0 mc/mM), sodium pyruvate-3-^{14}C (5.44
        mc/mM) and $H_3^{32}PO_4$ in 0.02M HCl (10 mc/ml) were purchased from
        New England Nuclear (Boston, Massachusetts).

    4. <u>Nembutal</u> (Sodium pentobarbital) was purchased from Abbott
        Laboratories (Chicago, Illinois).

    5. <u>Ninhydrin</u> spray reagent in disposable aerosol cans was purchased
        from E. Merck (Darmstadt, Germany) or prepared as described in
        the following section.

B. <u>Preparation of Special Solutions</u>

    1. <u>Ninhydrin</u> (for use as a spray in detecting AEP) was prepared
        as follows: 0.3 g ninhydrin (triketohydrindene hydrate) was

dissolved in 95 ml isopropanol, 5 ml collidine and 5 ml acetic acid.

2. <u>Ammonium Molybdate - Stannous Chloride</u> spray (for detection of orthophosphate on chromatograms) was prepared as follows:

   a) 1% Ammonium molybdate solution (5.0 g ammonium molybdate/ 500 ml H_2O)

   b) 1% $SnCl_2$ in 10% HCl (2.0 g $SnCl_2$/200 ml of 10% HCl).
   The chromatogram is sprayed with a), dried and then sprayed with b). Orthophosphate gives a blue spot.

3. <u>Ammonium Molybdate Solution</u> (for analysis of orthophosphate) was prepared as follows: 18.8 g of ammonium molybdate (Mallinckrodt, Los Angeles, Calif.) were dissolved in 300 ml of distilled H_2O, 150 ml of 98% H_2SO_4 were slowly added, the solution cooled over an ice bath and then diluted to a total of 500 ml solution with distilled H_2O.

4. <u>Standard Phosphate Solution</u> (1.0 mg P_2O_5/ml) was made as follows (65): 1.9157 g of KH_2PO_4 (Mallinckrodt, Los Angeles, Calif.) previously dried at 105° for 1 hr was dissolved in H_2O in a 1.0 liter volumetric flask and the flask brought to volume with distilled H_2O. A 10.0 ml aliquot of this solution was then diluted to 100.0 ml in a volumetric flask and this standard solution contained 0.1 mg P_2O_5/ml. A curve of <u>P_2O_5 concentration</u> vs. <u>absorbance</u> at 430 mμ was constructed (after mixing appropriate amounts of P_2O_5 with ammonium molybdate solution) according to the method of Bernhart and Wreath (65).

This curve followed the Beer-Lambert Law at dilute concentrations (2-30 µg P_2O_5/ml).

5. <u>Diazomethane</u> was freshly prepared from Diazald (N-methyl-N-nitroso-p-toluenesulfonamide), Aldrich Chemical Co., (Milwaukie, Wisconsin) as follows: 21.5 g (0.1 mole) of Diazald in 200 ml ether is added to a 500 ml erlenmeyer flask containing 25 ml 95% ethanol, 5 g KOH and 8 ml H_2O. This flask is then connected (by a piece of glass tubing and corks) to a receiving flask (containing about 50 ml ether) in an ice-acetone bath. Diazomethane is generated by heating the flask containing the Diazald at about 65°C. This process yields about 3 g of diazomethane.

6. <u>Krebs-Ringer-phosphate buffer</u> (for the incubation medium for the *in vitro* biosynthetic studies) was freshly prepared immediately before use from standard salt solutions as follows: 100 parts of 0.90% NaCl (0.154 M; 9.00 g/l),

   4 parts of 1.15% KCl (0.154 M; 11.48 g/l),

   3 parts of 1.22% $CaCl_2$ (0.11 M; 12.21 g/l),

   1 part of 3.82% $MgSO_4$.$7H_2O$ (0.154 M; 37.96 g/l),

and 20 parts of 0.1 M phosphate buffer, pH 7.4 (17.8 g Na_2 HPO_4.$2H_2O$, 20 ml 1N HCl, all diluted to 1.0 liter) were added together and thoroughly mixed.

7. <u>$NaHCO_3$ - Krebs buffer</u> (for incubation medium of the ^{32}P-orthophosphate biosynthetic experiment) was prepared to contain the following constituents at the indicated concentrations:

120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $Mg(OAc)_2$ and 0.2% $NaHCO_3$. These are thoroughly mixed with the $NaHCO_3$ being added immediately prior to use.

8. <u>Sodium-phosphate buffer</u> (for red blood cell ghost preparation) was prepared as follows: 5.3 ml of solution A (27.8 g NaH_2PO_4 per liter) is added to 94.7 ml of solution B (53.65 g of $Na_2HPO_4.7H_2O$ per liter). This is diluted to 4.0 liter and yields a pH 8.0, 0.005 M buffer.

C. <u>Instrumentation and Methods</u>

1. <u>Infrared</u> (IR) spectra were taken with a Perkin-Elmer 337 grating spectrophotometer.

2. A Perkin-Elmer 202 <u>ultraviolet-visible</u> <u>spectrophotometer</u> was used to assay for the phosphoammonium-molybdate complex at 430 mμ.

3. <u>Nuclear magnetic resonance</u> (NMR) spectra were determined with a Varian HA-100 spectrometer in the solvents specified. Chemical shifts are expressed in Tau values (τ) relative to tetramethylsilane (TMS).

4. <u>Mass Spectra</u> were obtained with a CEC Model 21-110B high resolution mass spectrometer at 100° probe temperature and 70 eV.

5. <u>Amino acid analyses</u> were done on a Beckman 120C Analyzer using the method of Moore et al. (66).

6. <u>Centrifugation</u> was done on a Sorval superspeed RC2-B automatic

refrigerated centrifuge.

7. <u>Melting points</u> were taken on a Thomas microscope hot stage and are uncorrected.

8. <u>Gas-liquid Chromatography</u> (GlC) was carried out on a F and M Model 810 gas chromatograph with a flame ionization detector.

Two different <u>columns</u> were found to be especially suitable: A) a prepoured glass column (6 ft x 0.25 in.) with 10% GE SE 30 (methyl silicone) on A-W Chromosorb G (Applied Science, Inglewood, California), and B) a glass column (8 ft x 0.25 in.) with 0.75% Hi-EFF-1 BP (diethylene glycol succinate), 0.125% EGSS-X (methyl organo-silicone) and 0.1% 144-B (phenyldiethanol-amine) on Gas-Chrom Q (materials for this column were purchased from Applied Science).

<u>Operating conditions</u> were as follows unless otherwise stated: H_2 pressure of 18 psig; air pressure of 20 psig; N_2 flow rate of 60 cc/min: injector temperature of 220-240°: flame ionization detector temperature of 315-390°.

9. <u>Radioactivity</u> was determined by liquid scintillation spectrometry in vials containing 10 ml toluene: ethanol (2:1) and scintillators (3.92 g PPO and 0.08 g MSB per liter, New England Nuclear, Boston, Massachusetts) with a Packard Tri-Carb Model 3002 Liquid Scintillator spectrometer or on planchets in a Nuclear-Chicago low background, gas flow counter.

10. <u>Elemental analyses</u> were performed by Spang Microanalytical Laboratory (Ann Arbor, Michigan) or by Heterocyclic Chemical

Corporation (Harrisville, Missouri).

11. <u>Ion exchange chromatography</u> was accomplished using Dowex 1-X8, 200-400 mesh, acetate form and Dowex 50W-X4, 200-400 mesh, H^+ form. The Dowex 1 column was changed from the chloride form into the acetate form by eluting with saturated potassium acetate solution until chloride ion no longer passed out in the effluent (as determined by no AgCl precipitating when $AgNO_3$ was added to the effluent). The Dowex 50W column was prepared by treating the resin in cycles with 2N HCl, distilled H_2O and 2N NaOH until the fines and the contaminating yellowish material were no longer present. The Dowex 1 column was eluted with distilled H_2O and the Dowex 50W column with 0.1 N HCl.

The Dowex 50W columns were regenerated after use by eluting with 1 N NaOH (containing 2 ml Brij 35 detergent per liter of NaOH) followed by elution with distilled H_2O. The column was then put back into the H^+ form by treating it with excess 1 N HCl.

12. <u>Thin layer chromatography</u> (tlc) was done on silica gel and alumina plates and on commercial 160 micron cellulose thin layer plates (Eastman Chromagram, Rochester, New York) in the following solvent systems:

    a) n-butanol: acetic acid: H_2O (3:1:1, v/v).

    b) isopropanol: formic acid: H_2O (2:1:1, v/v).

    c) acetone.

        d) benzene: methanol (2:1, v/v).

13. <u>Paper chromatography</u> (ascending and descending) was done on

    Whatmann No. 1 paper in the following solvent systems:

        a) 1-butanol: acetic acid: H_2O (4:1:4, v/v).

        b) isopropyl alcohol: formic acid: H_2O (8:1:1, v/v).

        c) methanol: pyridine: H_2O (17:1:5, v/v).

        d) pyridine: H_2O (65:55, v/v).

14. <u>Qualitative microphosphorus test</u> - This test was developed

    after the method of Hirata and Appleman (67) to assay for

    phosphonate-phosphorus in a sample which was negative for

    orthophosphate on HBr hydrolysis (no absorbance at 430 mμ

    after the addition of ammonium molybdate solution).

    1-2 mg of material to be assayed is placed in a test tube

    with 0.5 ml 70% $HClO_4$. This is heated in a sand bath until

    the mixture boils and digests the material. The digestion is

    complete when the particulate matter is no longer present and

    the solution becomes colorless. Then 2 ml of ammonium

    molybdate solution and 2 ml of acetone (to enhance development

    of the yellow color) are added. If phosphorus was present in

    the original sample, the solution turns yellow (absorbs at

    430 mμ) due to formation of the phosphoammonium-molybdate

    complex.

15. <u>Hydrolyses</u> were performed by refluxing the appropriate

    materials (tissues, tissue extracts and authentic compounds)

    in 24% aqueous HBr for a minimum of 6 hr. Control studies

indicated that the conditions of hydrolysis yielded 100%
hydrolysis of a test compound (creatine phosphate) and no
hydrolysis of AEP (with up to 72 hrs. of hydrolysis). The
amount of phosphorus was determined by assaying for inorganic
phosphate as its phosphoammonium-molybdate complex (see
following section for details of this assay).

16. A modification of Quin's method (15) for detecting C-P
phosphorus was developed and used initially to screen some
tissues for phosphonate phosphorus. This procedure was carried
out as follows:

Hydrolyses:

A. Tissue or tissue extracts (20-50 mg) were hydrolyzed
under reflux for at least 6 hr. in 5 ml 48% HBr.

B. Condensors were washed down with about 45 ml distilled
H_2O which was added to the hydrolysate.

C. Hydrolysates were filtered through glass wool and ash-
less filter paper into 100 ml volumetric flasks. When
the hydrolysate had color, a pinch of processed charcoal
was added before filtering. About 10 ml H_2O was used
to wash out the hydrolysis flask and this was also
filtered into the volumetric flask. The 100 ml volu-
metric flask is then brought to volume with distilled
H_2O.

D. A 5 ml aliquot of the above was transferred to a 25 ml
volumetric flask, 2 ml ammonium molybdate solution was

added and acetone was added to volume.

E. The color developed for 30 min. and was read at 430 mμ
against a blank [2 ml ammon. molyb. sol^{n} + 5 ml HBr
solution (10 ml 48% HBr/200 ml aq. solution) + 18 ml
acetone].

Oxidations:

A. Oxidation was performed with 4 ml boiling 70% $HClO_4$
in a flask containing tissue or tissue extracts
(20-60 mg).

B. Oxidation was continued until the tissue extract dis-
appeared and a colorless solution resulted.

C. The oxidation mixture was then filtered through glass
wool and ashless filter paper into a 100 ml volumetric
flask. The oxidation flask was washed with about 50
ml H_2O and filtered into the volumetric flask, which
was then brought to volume with distilled H_2O.

D. A 5 ml aliquot of the above was transferred to a 25 ml
volumetric flask, 2 ml ammonium molybdate solution was
added and acetone was added to volume.

E. The color developed for 30 min. and was read at 430 mμ
against a blank [2 ml ammon. molyb. sol^{n} + 5 ml $HClO_4$
solution (8 ml 70% $HClO_4$/200 ml aq. solution) + 18 ml
acetone].

The difference between total phosphorus (as determined
by $HClO_4$ oxidation) and hydrolyzable phosphorus was

assumed to be phosphonate (C-P) phosphorus (15).

D. Tissues and tissue preparation

1. Human tissues - The brain, liver and skeletal muscle were taken from a 38-year-old Caucasian male who died as a result of a crushing chest injury. Postmortem examination showed no evidence of systemic disease. These tissues were removed, frozen and stored (3 years) until used. The heart was from a 27-year-old Caucasian female who died of a heroin overdose. This tissue was removed directly after autopsy and immediately processed. The blood was from five individuals and was stored under refrigeration prior to use.

2. Rats used in the biosynthetic studies (Chapter V) were young, male Sprague-Dawley rats weighing approximately 125 g. These rats were housed in their cages for at least 3 days after their arrival and were given food (Purina Laboratory Chow) and H_2O *ad libitum.*

3. Rat liver slicing for the *in vitro* biosynthetic studies (Chapter V) was accomplished using a Stadie-Riggs microtome (68). This instrument allowed production of liver slices approximately 0.5 mm in thickness, which is optimum for preserving cell integrity yet allows free diffusion between the tissue and incubation medium (69).

4. Tissue homogenization was done either with a variable-speed Waring blender or with a Potter-Elvehjem homogenizer.

5. Freeze-drying was performed on certain tissues (human liver

and brain) using standard lyophilization techniques.

Chapter III - DEVELOPMENT OF A NEW ASSAY TECHNIQUE FOR THE DETECTION
OF AEP

A. Introduction

As mentioned in the introductory chapter, when this study began no
adequate assay for the specific and unambiguous detection of AEP was
available. Therefore, the development of such an assay which would be
applicable to tissues and tissue extracts was undertaken.

The techniques of gas-liquid chromatography (glc) and mass spec-
trometry have been successfully utilized for the detection and un-
ambiguous characterization of small amounts of many natural products
(70,71). These two highly sensitive, vapor phase techniques were
utilized in the new AEP assay. Since AEP is polar and nonvolatile
(2,4), it was necessary to convert AEP into a less polar (and more
volatile) derivative.

B. Derivatization of AEP

The problem of high polarity and low volatility of amino acids
can be overcome by acylation of the amino group and esterification

of the carboxylic acid hydroxyl group(s). Figure 5 illustrates the sequential acylation (acetylation) and esterification (methylation) reactions which successfully converted AEP to a volatile derivative (dimethyl 2-acetamidoethylphosphonate) which was suitable for glc and mass spectrometric analysis.

2-Acetamidoethylphosphonic Acid. To 42 mg of AEP was added 8 ml of acetic acid-acetic anhydride (1:1, v/v). The resulting mixture was stirred with a magnetic stirrer at room temperature until complete solution was obtained (approximately 2 hr; the rate of this acylation can be increased by heating the reaction mixture). The solution was then filtered and the volatile reagents were removed under reduced pressure (Rinco evaporator). The resulting residue was dissolved in a minimum amount of hot methanol and the solution was filtered. On cooling the filtrate to room temperature crystallization occurred, yielding 32 mg (75%) of 2-acetamidoethylphosphonic acid; mp 172-180° which was characterized by the following data:

1. NMR: (Me$_2$SO-d$_6$)*: 2.19, broad (NH); 6.82 multiplet (N-CH$_2$); 8.24, singlet (CH$_3$C=O); 8.32, multiplet (P-CH$_2$).

2. IR: (KBr): 1590 cm^{-1} (C=O).

3. Elemental Analysis: Calculated for C$_4$H$_{10}$NO$_4$P·0.5H$_2$O :
C,27.3%; H, 6.26%; N, 7.96%. Found:
C,27.5%; H, 6.27%; N, 7.90%.

* Chemical shifts are given in Tau (τ) values relative to TMS.

$$\underset{\text{H}_2\text{NCH}_2\text{CH}_2\overset{\displaystyle\overset{O}{\|}}{\underset{\displaystyle\underset{OH}{|}}{P}}\text{OH}}{} \qquad \text{(AEP)}$$

HOAc: Ac_2O (1:1)

$$\text{CH}_3\overset{\displaystyle\overset{O}{\|}}{\text{C}}\text{NHCH}_2\text{CH}_2\overset{\displaystyle\overset{O}{\|}}{\underset{\displaystyle\underset{OH}{|}}{P}}\text{OH} \qquad \begin{array}{l}\text{(2-Acetamido-}\\ \text{ethylphosphonic Acid)}\end{array}$$

$CH_2N_2(Et_2O)$,MeOH

$$\text{CH}_3\overset{\displaystyle\overset{O}{\|}}{\text{C}}\text{NHCH}_2\text{CH}_2\overset{\displaystyle\overset{O}{\|}}{\underset{\displaystyle\underset{OCH_3}{|}}{P}}\text{OCH}_3 \qquad \begin{array}{l}\text{(Dimethyl 2-Acetamido-}\\ \text{ethylphosphonate)}\end{array}$$
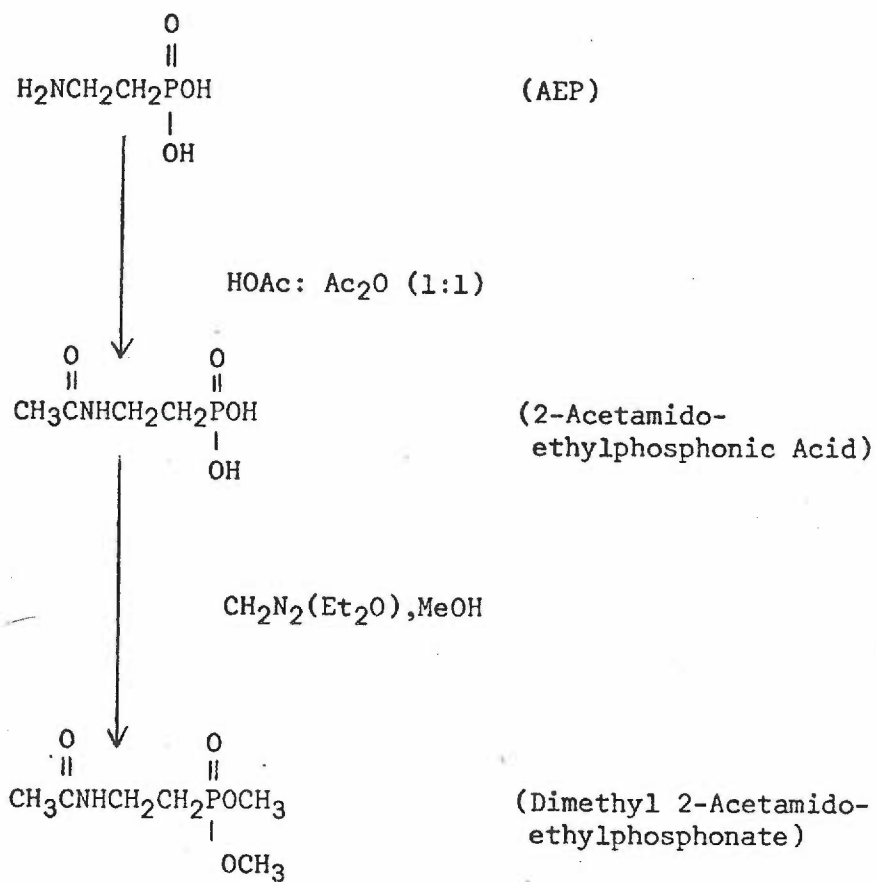
Figure 5 - Derivatization of AEP

Dimethyl 2-acetamidoethylphosphonate. To a solution of 30 mg of 2-acetamidoethylphosphonic acid in 5 ml of methanol was added excess freshly prepared diazomethane in ether. The solvents were evaporated using a stream of nitrogen to yield 26 mg (86%) of a viscous colorless liquid which was characterized as dimethyl 2-acetamidoethylphosphonate by the following data:

1. NMR: $(CDCl_3)^*$: 3.44, broad (NH); 6.24, doublet (6H, OCH_3); 6.50, multiplet ($N-CH_2$); 7.98, multiplet ($P-CH_2$); 8.20, singlet ($CH_3C=O$)

2. IR: (smear on salt plates): 1655 cm^{-1}(C=O).

3. Elemental analysis: Calculated for $C_6H_{14}NO_4P$:

    C,36.9%; H, 7.19%; N, 7.18%. Found:

    C,36.9%; H, 7.37%; N, 7.08%.

4. Mass Spectrum: See Figure 6.

C. GLC of Dimethyl 2-Acetamidoethylphosphonate

1. Chromatographic Materials

Once the AEP derivative had been made and fully characterized it was necessary to determine its suitability for gas liquid chromatographic analysis. Several different liquid and solid supports were investigated in order to find both polar and a nonpolar system which

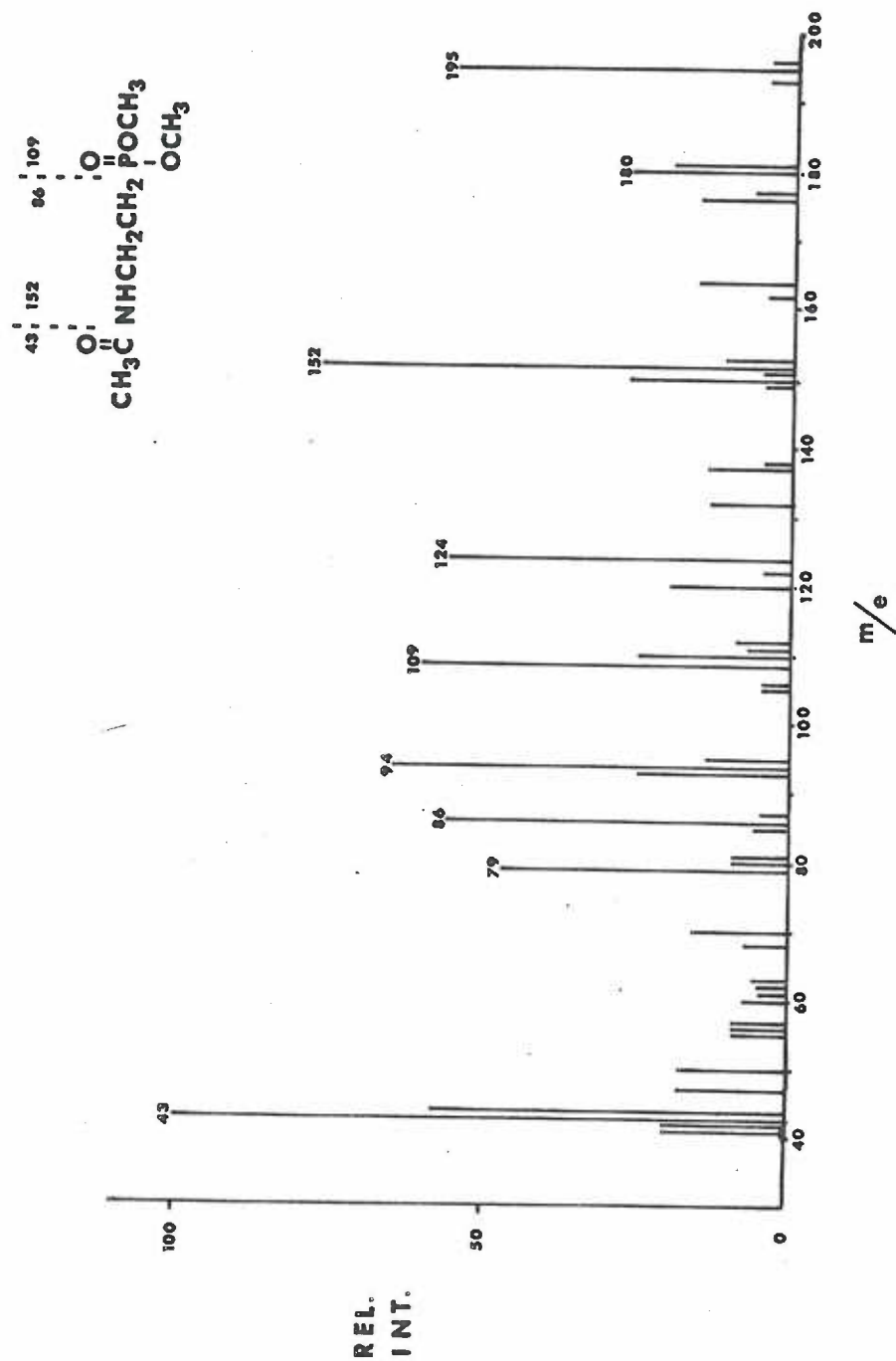* Chemical shifts are given in Tau (τ) values relative to TMS.

Figure 6 - Mass Spectrum of Dimethyl 2-acetamidoethylphosphonate

might be utilized in the new assay being developed. This was done to have two types of columns with differing separation properties for detecting and isolating the AEP derivative. After investigating several systems two columns were found to be particularly suitable for glc of the AEP derivative: A) 10% GE-SE 30 on A-W chromosorb G (a nonpolar system), and B) 0.75% Hi-EFF-1BP, 0.25% EGSS-X and 0.1% 144B on Gas Chrom Q (see Materials and Methods for more information on this fairly polar system). Typical chromatograms of a chloroform solution of dimethyl 2-acetamidoethylphosphonate on columns A and B are shown respectively in Figures 7 and 8. Table I gives retention times for the AEP derivative on columns A and B at several different temperatures. The operating conditions are as described in Materials and Methods (Chapter II).

2. Sensitivity

Several variables affect the sensitivity of the gas chromatograph. For example, H_2 pressure, air pressure and N_2 flow are all important. These variables were optimized for greatest sensitivity through experimentation and resulted in those conditions listed in Materials and Methods. Another variable which had a tremendous effect on sensitivity was the temperature of the flame ionization detector. A standard solution of methyl tetradecanoate was made and initially used to test the sensitivity of the detector. It was found that 8 nanograms of methyl tetradecanoate gave an easily detectable and symmetrical peak at 290° detector temperature. However, under these conditions microgram quantities of the AEP derivative were necessary
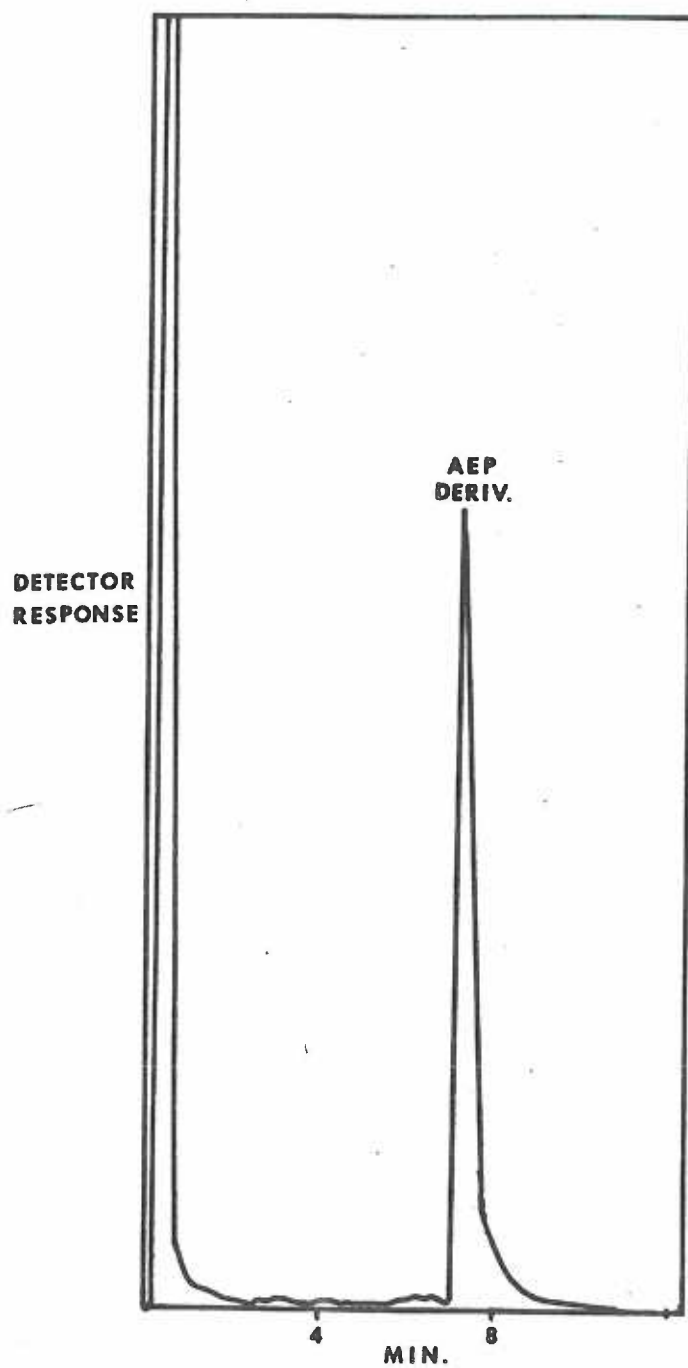
Figure 7 - Gas Chromatogram of Dimethyl 2-Acetamidoethylphosphonate at 180° on the 10% GE SE30 column. See Materials and Methods for operating details.
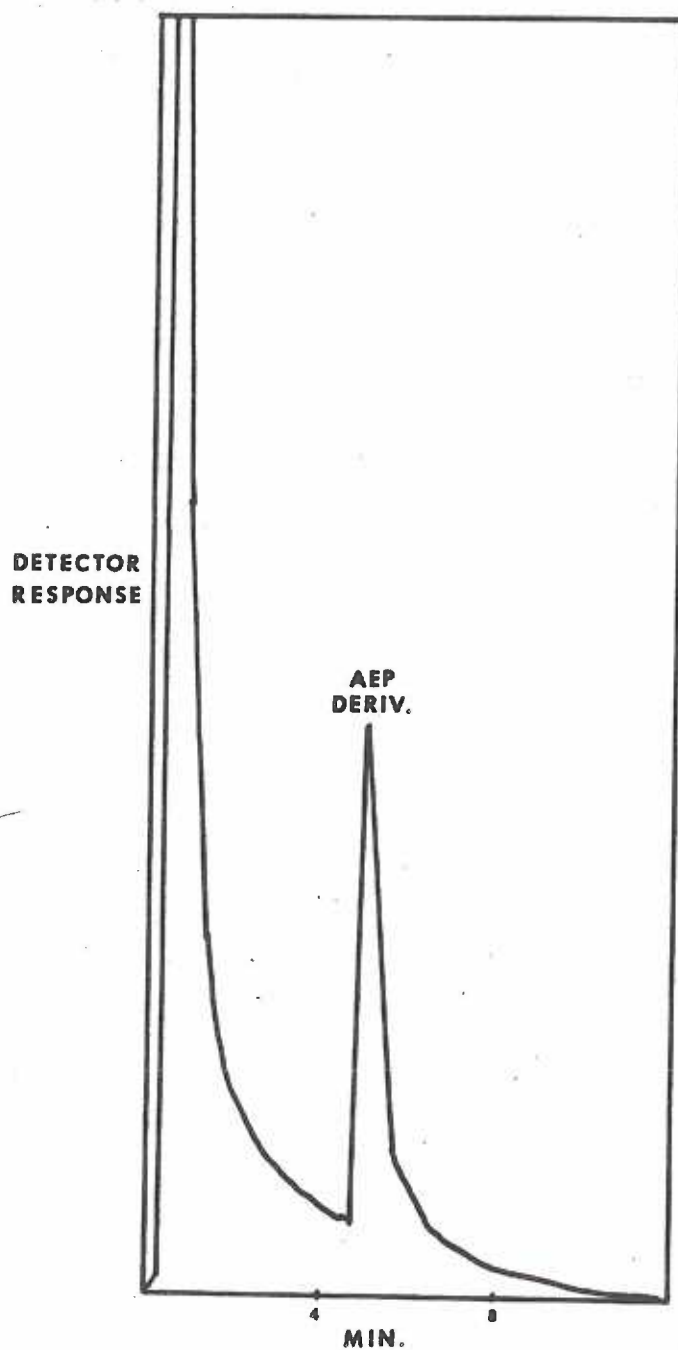
Figure 8 - Gas Chromatogram of Dimethyl 2-Acetamidoethylphosphonate
at 195° on the 0.75% Hi-EFF-1BP, 0.25% EGSS-X and 0.1%
144B column. See Materials and Methods for operating
details.

Table I - Retention times (in minutes) of Dimethyl 2-acetamidoethyl-
phosphonate on two columns at varying temperatures. See
Materials and Methods for details of operating conditions.

TEMP. °C	Column A - 10% GE SE 30 on A-W Chromosorb G	Column B - 0.75% Hi-EFF-1BP, 0.25% EGSS-X, and 0.1% 144B on Gas Chrom. Q.
150°		31.0 ± 1.0 min.
170°	9.6 ± 0.4 min.	
175°		10.5 ± 0.4 min.
180°	7.4 ± 0.4 min.	9.7 ± 0.4 min.
185°		7.0 ± 0.4 min.
190°	5.6 ± 0.4 min.	5.7 ± 0.4 min.
195°		4.9 ± 0.4 min.
200°	4.4 ± 0.4 min.	

for its detection. It was then hypothesized that the temperature of
the flame ionization detector was hot enough to ionize the methylated
fatty acid near maximally but not the phosphonate. Therefore, a
study of detector response versus detector temperature was undertaken
using a constant sample size (0.25 μg dimethyl 2-acetamidoethylphos-
phonate). Table II gives the results of this study. It can be seen
that as the temperature of the detector increased from 290° to 390°,
the sensitivity of the detector to the derivatized phosphonate in-
creased nine-fold (whereas the sensitivity to methyl tetradecanoate
only increased two-fold). These findings support the above mentioned
hypothesis that a hotter flame ionization detector system was
necessary to maximize the ionization of the phosphonate. Using a
detector temperature of 390° and the other conditions as described
in Materials and Methods, it was possible to detect from 25-50 nano-
grams of the AEP derivative. Because 10 μl injections were generally
used, this corresponds to a sensitivity of 2.5-5.0 μg AEP derivative
per ml.

3. Quantitation of the AEP Derivative

The new assay was developed to determine both the presence
and the concentration of AEP in tissues and tissue extracts. The
amount of AEP in the sample being assayed was usually determined by
integrating the appropriate peak for the AEP derivative and by com-
paring this to the areas of the peaks resulting from injections of
known amounts of the AEP derivative. Integration was done manually
by approximating the peak by a triangle and then determining the area

Table II - Study of GLC detector response versus detector temperature
with a constant sample size (0.25 µg AEP derivative). See
Materials and Methods for details of operating conditions.

Flame Ionization Detector Temperature	Detector Response [*]
290°	30 sq. units
330°	74 sq. units
360°	156 sq. units
390°	270 sq. units

[*] Detector response was determined by the area
of the peak corresponding to the AEP deriva-
tive. This area was calculated by approxi-
mating the peak by a triangle and then deter-
mining its area.

of the triangle. A study was done on the detector response to varying amounts of dimethyl 2-acetamidoethylphosphonate to see if this response was linear, a necessary condition if peaks of varying sizes were to be compared to a given amount of the AEP derivative. Figure 9 is a graph of GLC detector response (area) versus amount of AEP derivative. This graph indicates that the detector response was nearly linear between 0.25 µg and 1.0 µg of the AEP derivative.

Alternatively the amount of AEP in a sample was determined through "spiking" with an internal standard of dimethyl 2-acetamidoethylphosphonate. A sample was assayed and then the same amount of sample containing a known amount of AEP derivative was injected. Because the difference between the appropriate peak areas in these two injections was due to a known amount of AEP derivative, the concentration of endogenous AEP in the sample can be easily calculated. Although this method is quite laborious, it probably yields more accurate results than comparison of peak areas to the standard curve of detector response to varying amounts of AEP (Figure 9). Furthermore, this method has the added advantage of determining whether the known AEP derivative exactly co-chromatographs with the putative AEP peak.

4. Preparative GLC

Preparative gas-liquid chromatography was carried out under the same conditions as analytical glc except that a five-to-one stream splitter was inserted on the chromatograph. This splits the carrier gas into two fractions, with one-sixth going to the flame ionization detector and the remainder going to the heated (approx.
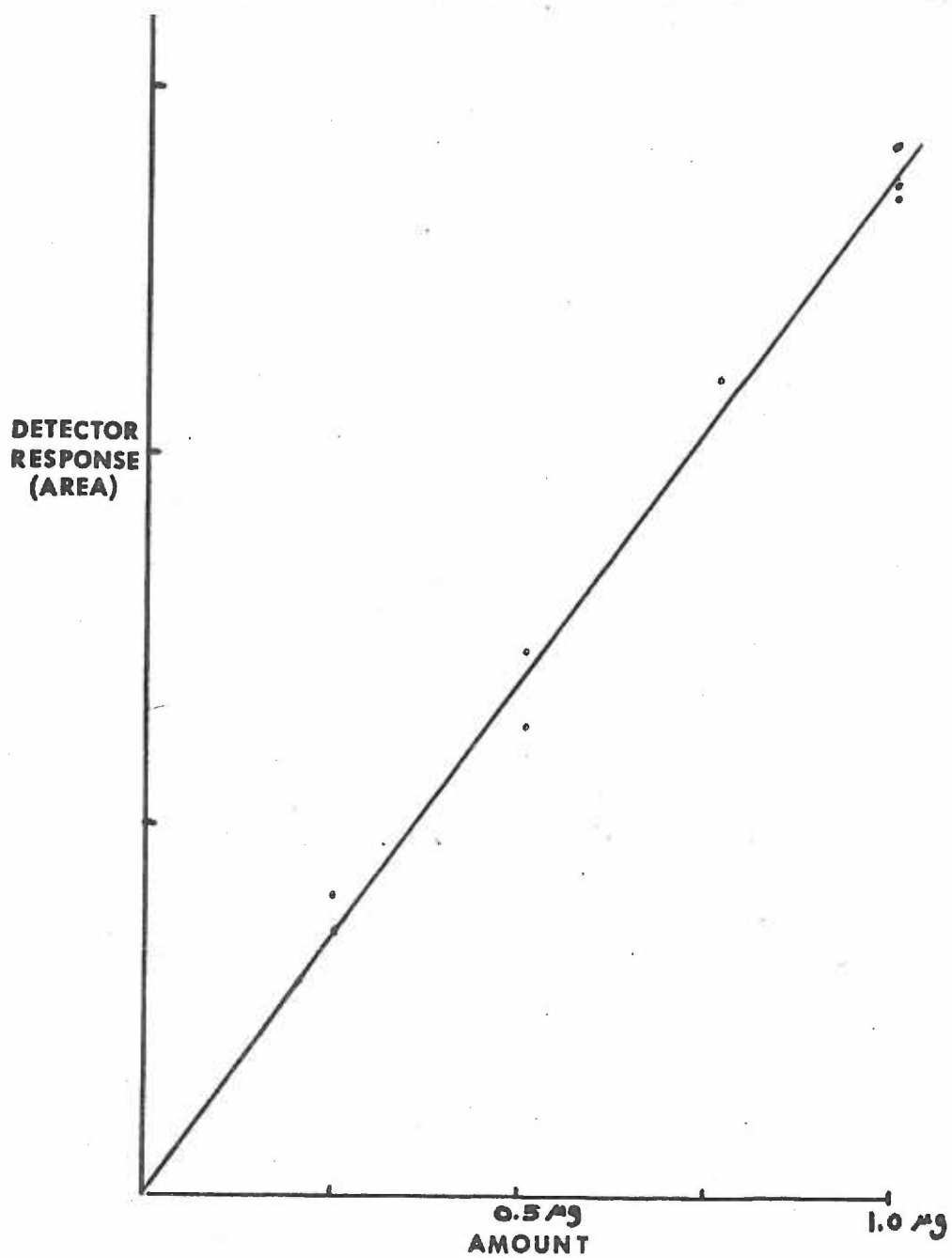
Figure 9 - GLC Detector Response (area) versus Amount of AEP derivative.

GLC conditions are as described in Materials and Methods

(Chapter II).

240°) effluent port. The appropriate peak was collected by holding a piece of glass tubing (4 in. x 1/4 in.) over the end of the effluent port. The AEP derivative on exiting from the effluent port condenses in the glass tubing. Using this method an authentic sample of the AEP derivative was chromatographed, collected and identified by mass spectrometry. For collection of very small samples (2-10 μg) a longer glass tube (9 in.) was used. This tube was jacketed by a larger diameter tube filled with powdered dry ice which acted as a condensor. This system allowed the collection of a 2 μg sample of the AEP derivative.

D. Model Studies

To assess the suitability of the assay technique for detection of AEP in complex mixtures, a model study was carried out. A mixture of 2.0 mg of AEP and 30.0 mg of each of 5 amino acids (Glu, Val, Phe, Thr, and Asp) in 6 ml of acetic acid-acetic anhydride (1:1, v/v) was stirred for 3 hr. The solvent was evaporated under reduced pressure, the residue was dissolved in 50 ml of methanol and a 5 ml aliquot was treated with excess diazomethane in ether. The resulting mixture was evaporated, the residue was dissolved in chloroform and subjected to gas-liquid chromatographic analysis using the 10% GE SE30 column (column A) at column temperatures of 170°-200° (isothermal) and the conditions described in Materials and Methods (Chapter II). The AEP derivative was readily separated from the amino acid derivatives at column temperatures of 170°, 180° or 200° exhibiting retention times of 9.6, 7.4, and 4.4 min., respectively. Figure 10 is a gas-liquid
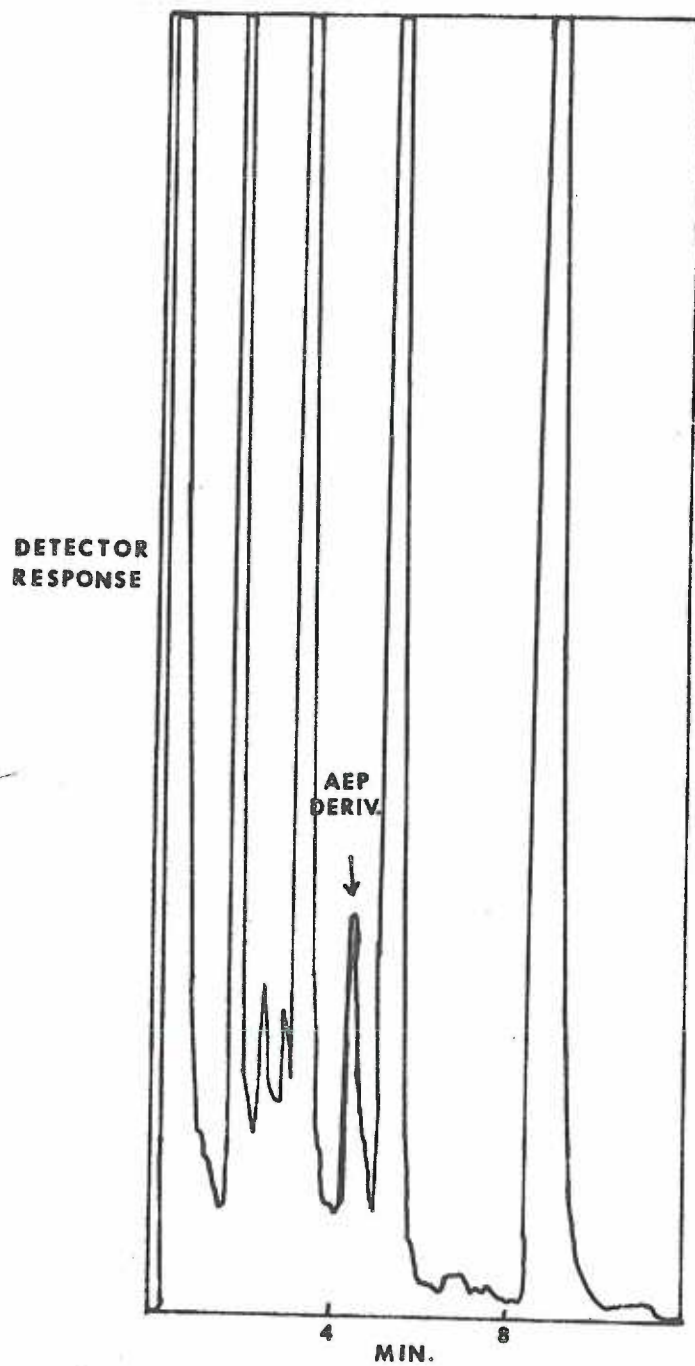
Figure 10 - Gas-liquid chromatogram of mixture of derivatized AEP and
amino acids on a 10% GE SE30 column at 200°. Other condi-
tions are as indicated in Materials and Methods.

chromatogram of the derivatized mixture. Comparison of this chromatogram with those obtained using standard solutions of the AEP derivative (see previous section on Quantitation) indicated that essentially quantitative conversion of AEP to its volatile derivative had been achieved.

E. Assay of Tissues for AEP

Tissues and tissue extracts which were to be assayed for AEP were subjected to the procedure depicted schematically in Figure 11. Tissues were hydrolyzed for at least 6 hr. in 24% HBr[*] and the hydrolysate defatted by extraction with $CHCl_3$. An aliquot of the defatted hydrolysate at this point can be assayed for AEP by the automatic amino acid analyzer (5,9,20,30,45). Figure 12 shows an amino acid analysis chromatogram of the hydrolysate of a hexane soluble fraction of human brain which contained AEP. Using this technique AEP can be detected at a concentration of 0.1 μM AEP per ml (12.5 μg/ml). Another aliquot can be derivatized as depicted in Figure 11 and then unambiguously assayed for the AEP derivative by combined glc-mass spectrometry. However, complex gas-liquid chromatograms usually result due to the complexity of the tissue hydrolysate. Figure 13 illustrates such a complex chromatogram obtained by glc of a derivatized hydrolysate of a

[*] See control studies on HBr hydrolysis in Chapter II, Materials and Methods.

Tissue fraction

$\downarrow$

hydrolysis, 24% HBr

$\downarrow$

Hydrolysate

$\downarrow$

$CHCl_3$

Sol.   Insol.

$\downarrow$

$HOAc:Ac_2O$ (1:1)

$\downarrow$

Acetylated derivative

$\downarrow$

$CH_2N_2(Et_2O),MeOH$

$\downarrow$

Derivatized sample
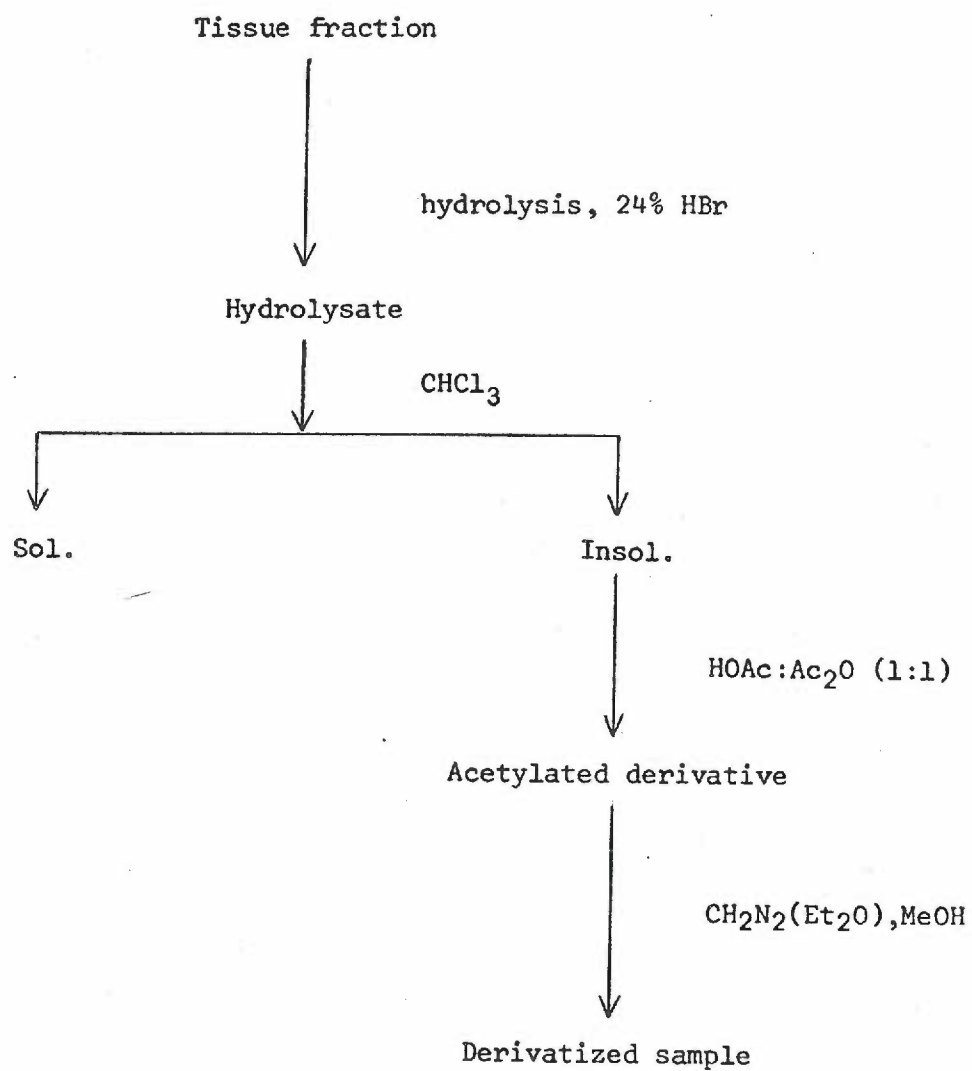
Figure 11 – Preparation of Tissue for AEP Assay

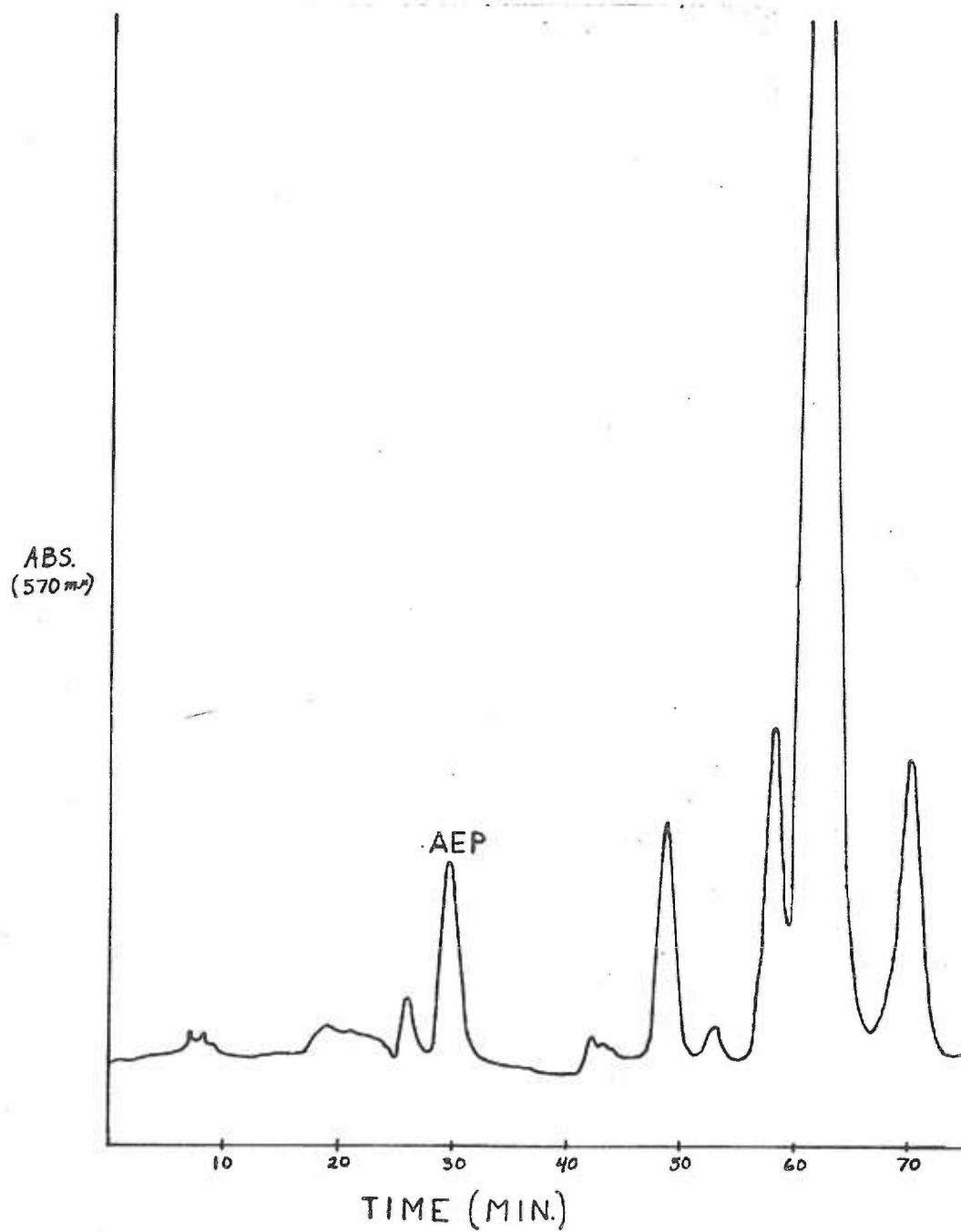Figure 12 - Automatic amino acid analysis chromatogram of hexane-
soluble human brain hydrolysate fraction. Details of
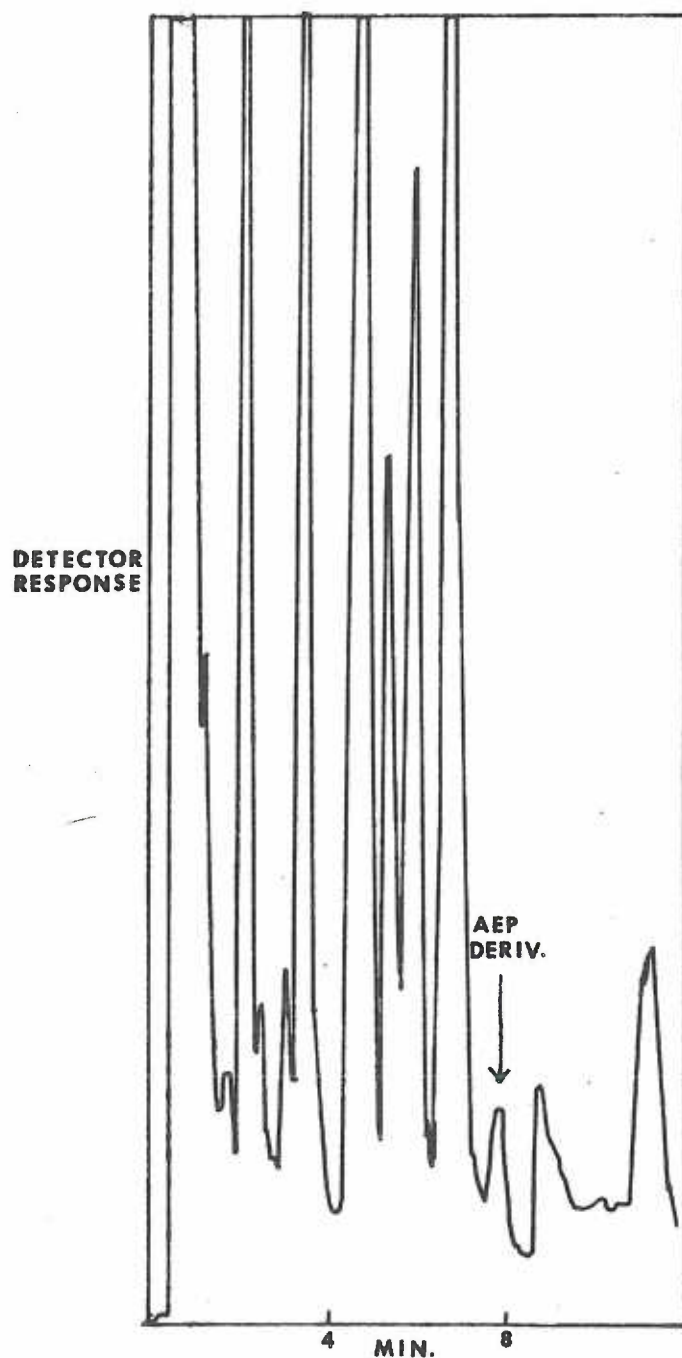conditions of analysis are given in Materials and Methods.

Figure 13 - Gas-liquid chromatogram of derivatized hydrolysate of a
polar fraction (hexane-methanol insoluble) of human brain
on a 10% GE SE30 column at 180°. Other conditions are as
indicated in Materials and Methods.

polar fraction (hexane-methanol insoluble) of human brain. It is difficult to isolate the AEP derivative by preparative glc from such a complex mixture. Therefore, two alternate purification and concentration steps were developed, one employing Dowex 50W ion exchange chromatography and the other preparative tlc on alumina or silica gel plates.

1. Dowex 50W Concentration. This step for the concentration of AEP in tissue hydrolysates involves a treatment of a defatted ($CHCl_3$ extracted) tissue hydrolysate on a 2 x 26.5 cm column of Dowex 50W-X4, 200-400 mesh, H^+ form. Approximately 2-3 g of tissue hydrolysate are put on the column in a minimum amount of 0.1 N HCl (approximately 2-4 ml). Elution is carried out with 0.1 N HCl and AEP (when present) elutes near the front (approximately 300-630 ml) with a few other acidic components, yielding a fraction greatly enriched in AEP (most of the other amino acids and unknown compounds in the hydrolysate are retained on the column at the low pH used for elution). This AEP-enriched fraction can then be assayed (after derivatization) by glc. Figure 14 is a glc chromatogram of a human brain hydrolysate fraction (hexane soluble) which was chromatographed on Dowex 50W prior to derivatization. It can be seen that this treatment yields a less complex chromatogram (compare to Figure 13) with a much larger concentration of the AEP derivative.

2. Preparative TLC Concentration. An alternate way of concentrating the AEP in tissue hydrolysates is through preparative thin layer
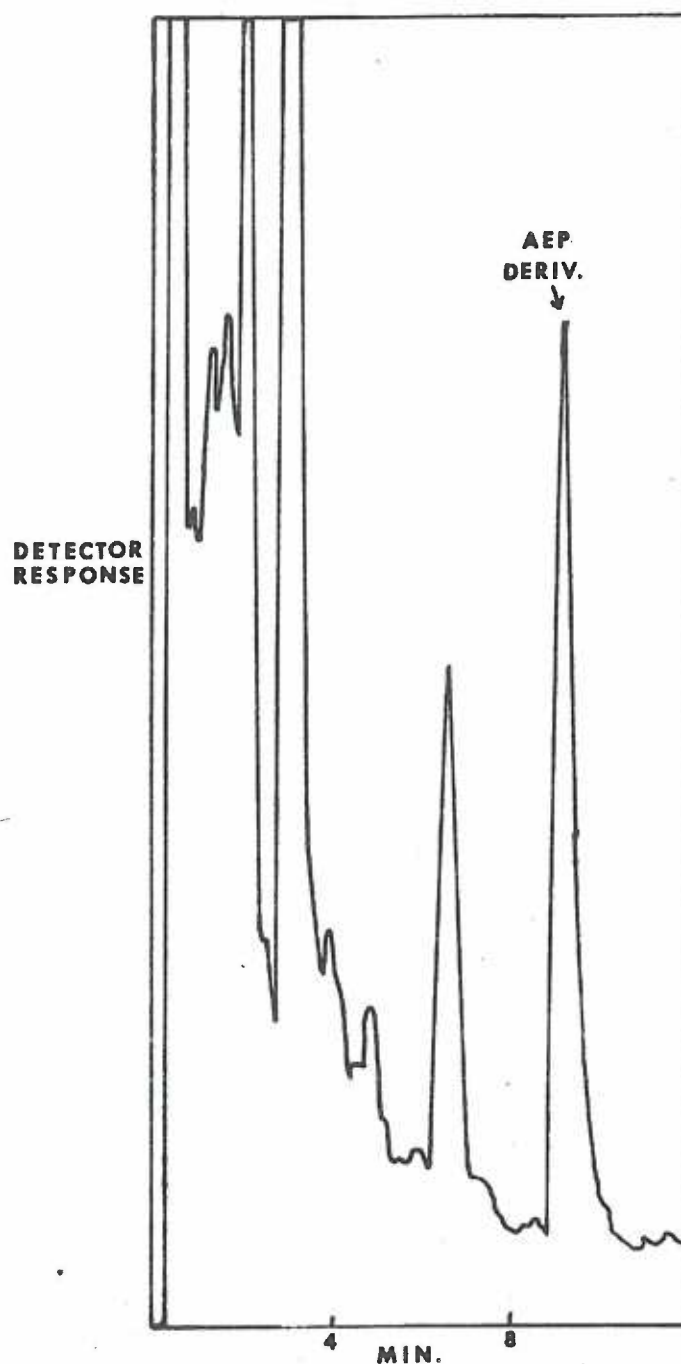
Figure 14 - Gas-liquid chromatogram of Dowex 50W-treated hydrolysate of
a human brain fraction (hexane soluble) on a 10% GE SE30
column at 170°. Other conditions are as indicated in
Materials and Methods.

chromatography. A derivatized tissue hydrolysate is subjected to
preparative tlc on 0.5 mm alumina plates. These plates are develop-
ed in a chromatographic tank with acetone as a solvent using an
authentic sample of dimethyl 2-acetamidoethylphosphonate as a
marker. Visualization is accomplished with iodine vapor. Dual
development in acetone results in an approximate Rf of 0.2. A band
(Rf 0.15-0.25) is removed from the plate and eluted with chloroform.
The chloroform eluate is then analyzed for the AEP derivative by
gas-liquid chromatography and mass spectrometry. Preparative thin
layer chromatography of derivatized tissue hydrolysates was also
accomplished using 1.0 mm thick silica gel plates with development
in benzene: methanol (2:1, v/v). Using this system the AEP derivative
exhibits an Rf value of approximately 0.4.

Chapter IV - <u>STUDIES ON THE OCCURRENCE, DISTRIBUTION AND CONCENTRATION</u>
<u>OF AEP IN HUMAN TISSUES</u>

A. <u>Introduction</u>

After the new assay method was developed and adequately tested, it
was used to assay for AEP in various human tissues and tissue extracts.

B. <u>Initial Demonstration of AEP in Human Brain (30)</u>

1. <u>Fractionation of Brain Tissue</u>. An adult human brain (see
    Chapter II for source of brain) was homogenized in a Waring
    blender and subsequently lyophilized. The lyophilized tissue
    (36.7 g) was extracted with 3.5 liters of redistilled hexane
    by shaking overnight on a mechanical shaker. The hexane
    solution was removed, dried over sodium sulfate, and the
    solvent was removed to yield 12.5 g of residue (nonpolar
    lipids). The hexane-insoluble residue was extracted using 3
    liters of methanol for 12 hr. on a mechanical shaker. Removal
    of solvent from the methanol extract produced 7.8 g of polar
    lipids (72,73). The hexane-methanol-insoluble material

(proteinaceous residue) comprised 14.3 g. Figure 15 depicts the method by which human brain and liver were extracted.

2. Analysis of Nonpolar Lipid (Hexane Soluble) Fraction for AEP. A portion (8 g) of the hexane-soluble material was hydrolyzed by heating for 19 hr. in 500 ml of 24% hydrobromic acid under reflux. The cooled hydrolysate was diluted with 500 ml of distilled water, filtered and then extracted with three 650 ml portions of chloroform to remove lipids. The aqueous portion of the hydrolysate was evaporated under reduced pressure to yield 3 g of residue. A small aliquot of this residue (30 mg) was assayed for inorganic phosphate (as described in Materials and Methods). Another aliquot (27 mg) was assayed for total phosphorus by oxidation with 70% $HClO_4$ (as described in Materials and Methods). This analysis indicated that approximately 24% of the phosphorus in the hydrolysate of hexane-soluble human brain was phosphonate phosphorus. The remainder of the 3 g of hydrolysate residue was fractionated on a 2 x 27 cm Dowex 1-X8, 200-400 mesh, column in the acetate form. Elution was carried out with distilled water and the compound of interest was eluted in the first 50 ml (fraction I). This fraction was ninhydrin positive and contained a compound which was indistinguishable from authentic AEP by descending paper chromatography in the following solvent systems:

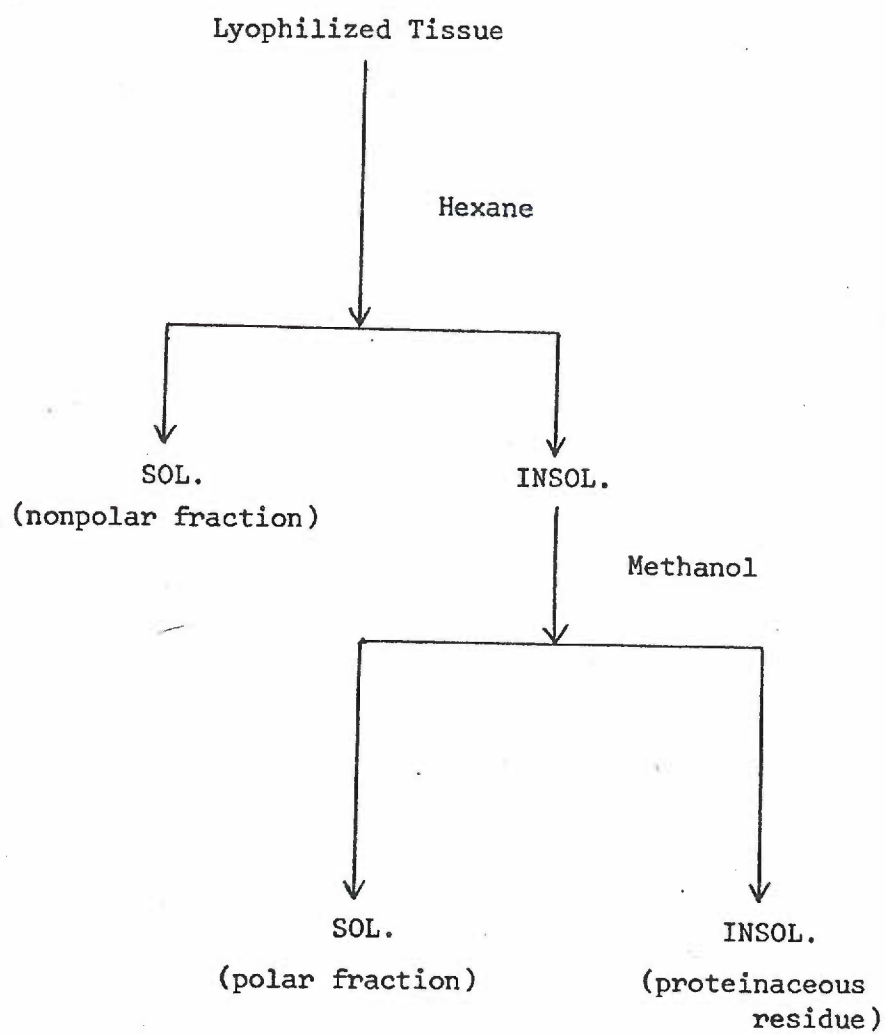(a) 1-butanol - acetic acid - H_2O(4:1:4, v/v), Rf 0.38.

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Figure 15 - Extraction Method used for Fractionating Human Brain and
Liver.

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(b) isopropyl alcohol-formic acid-H_2O(8:1:1), Rf 0.11.
Co-chromatography was also exhibited by ascending paper
chromatography in the following systems:

(c) methanol-pyridine-H_2O(17:1:5, v/v), Rf 0.43.

(d) pyridine-H_2O(65:55, v/v), Rf 0.42.

An aliquot of fraction I gave a negative test for inorganic
phosphate (74); a second aliquot was assayed by the microphos-
phorus test (described in Chapter II) and found to contain
phosphorus. These data indicated that fraction I contained
one or more compounds possessing a C-P bond.

A third aliquot of fraction I was brought up to 1 ml with
pH 2.2 citrate buffer and examined using the automatic amino
acid analyzer. A peak was eluted (28 min.) on a 55 cm column
which corresponded to that of authentic AEP (5,9,20,45).

The remainder of fraction I was derivatized as described in
Chapter III (acetylated and methylated), dissolved in $CHCl_3$
and examined by gas-liquid chromatography. A compound was
observed which exhibited retention times identical with those
of synthetic dimethyl 2-acetamidoethylphosphonate using six
different temperature regimes (170° isothermal; 180° isothermal;
150°, 4° rise/min; 150°, 6° rise/min; 150°, 10° rise/min; 150°,
15° rise/min.) on the 10% GE SE30 column and using the con-
ditions described in Materials and Methods. This compound was
isolated by preparative gas-liquid chromatography using a
column temperature of 170°. Eight 10-µl injections were

subjected to glc and the appropriate peak was collected. A
mass spectrum of the collected material exhibited a parent ion,
m/e 195.0650 (calculated for dimethyl 2-acetamidoethylphos-
phonate, 195.0654) and fragment ions identical with those
observed in a spectrum of the synthetic AEP derivative (see
Figure 6, Chapter III).

3. Analysis of Polar Lipid (Methanol Soluble) and Proteinaceous
    Residue (Hexane-Methanol Insoluble) Fractions for AEP.
    Samples (2 g) of each of the above fractions were hydrolyzed
    using 24% hydrobromic acid and extracted with chloroform as
    described for the hexane soluble fraction of human brain. The
    respective aqueous phases were evaporated to dryness and de-
    rivatized for gas-liquid chromatographic analysis. A peak
    corresponding to the AEP derivative was observed in the
    chromatogram for the proteinaceous residue (see Figure 13,
    Chapter III). This material was collected from the gas-liquid
    chromatographic column (thirteen 10-µl injections) and a mass
    spectrum was obtained which exhibited a molecular ion and a
    fragment pattern identical with that for an authentic sample
    of dimethyl 2-acetamidoethylphosphonate (see Figure 6, Chapter
    III). Gas-liquid chromatographic analysis of the derivatized
    mixture obtained from the polar lipid (methanol soluble)
    fraction hydrolysate did not reveal the presence of AEP (30).
    However, after a portion of this derivatized hydrolysate was

subjected to preparative thin layer chromatography on alumina (see Section E, Chapter III), the AEP derivative was found to be present in this purified fraction by glc and mass spectrometric analysis (31).

4. Presence of AEP in a Second Human Brain. A second human brain was processed as described for the first brain. Gas-liquid chromatographic analysis of the derivatized hydrolysates of all three fractions (hexane soluble, methanol soluble and hexane-methanol insoluble) indicated the presence of the AEP derivative. This was confirmed in one of the fractions (hexane-methanol insoluble) by high resolution mass spectrometry of a collected sample (parent ion, m/e 195.0656 as compared to m/e 195.0654 for an authentic sample of the AEP derivative).

C. Presence of AEP in Human Liver

A human liver was homogenized, lyophilized and extracted as indicated in Figure 15 (Chapter IV). The three resulting fractions were hydrolyzed with hydrobromic acid, defatted with chloroform and a portion of each was derivatized by sequential acylation and methylation (Figure 11, Chapter III). Preparative thin layer chromatography of these derivatized, tissue extract hydrolysates followed by gas chromatographic analysis indicated that AEP was present in the polar lipids (methanol soluble) and proteinaceous residue (hexane-methanol insoluble) fractions of human liver but not in the nonpolar lipid (hexane soluble)

fraction.

D. Concentration of AEP in Human Brain and Liver Fractions

An estimate of the concentrations of the AEP derivative in the human brain and liver fractions was made by comparing the areas of the appropriate gas chromatographic peaks with peak areas obtained using known amounts of dimethyl 2-acetamidoethylphosphonate (see section on Quantitation, C-3, Chapter III). The assumption was made that complete conversion of AEP into its derivative had been achieved (see Model Studies, Chapter III). The results, corrected to indicate concentration of AEP rather than of its derivative, are shown in Table III.

E. Analysis of Human Red Blood Cell Ghosts for AEP

Five units of human, packed, whole red blood cells (rbc) were processed for their cell membranes (ghosts) by a method modified after that of Steck et al. (75) and Dodge et al. (76). The cells were washed with isotonic saline (0.154 M) and the erythrocytes isolated by centrifugation for 20 min. at 1000 x g in a refrigerated (4°C) centrifuge (76). The isolated rbc's were lysed with 1 volume of distilled H_2O over a period of 2.5 hr. in the cold room. The lysate was then centrifuged for 20 min. at 15,000 x g and the supernatant was discarded. The pellet was suspended in several volumes of 0.005 M sodium phosphate buffer, pH 8.0, and again centrifuged for 20 min. at 15,000 x g. This

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Table III - Concentration of AEP in Human Brain and Liver Fractions.

Fraction	Concentration (mg AEP/g of fraction)	
	Brain	Liver
Hexane Soluble (nonpolar lipids)	0.4	not detected[*]
Methanol Soluble (polar lipids)	0.1	0.6
Hexane-Methanol Insoluble (proteinaceous residue)	1.0	1.0

[*] less than 50 µg AEP/g of fraction

process was repeated five times with 30 min. spins until the supernat-
ant was no longer extracting any color from the pellet. This resulted
in 7.7 g (wet weight) of pinkish-white rbc ghosts.

The rbc ghosts were hydrolyzed in 150 ml hydrobromic acid for 24
hr. The hydrolysate was filtered and extracted with four 150 ml
portions of chloroform. A 2.5% aliquot of the hydrolysate was assayed
for AEP by automatic amino acid analysis and indicated the presence of
AEP by a peak at 29 minutes.

Another aliquot (50%) of the rbc ghosts hydrolysate was derivatized
by sequential acetylation and methylation. Twenty-five percent of this
derivatized mixture was subjected to preparative thin layer chromato-
graphy on two, 8 in. x 8 in., 1.0 mm silica gel plates in benzene:
methanol (2:1). The thin layer plates were monitored by chromatograph-
ing authentic AEP derivative on the edges of the plates. Iodine vapor
was used to visualize the marker compound. The appropriate zones from
the thin layer plates were removed and eluted with about 15 ml methanol.
This solvent was evaporated under nitrogen and the residue dissolved
in 0.5 ml chloroform. Gas liquid chromatography on column B (see
Materials and Methods for details) indicated the presence of the AEP
derivative at two different column temperatures (170°, 150°). Further-
more, the putative AEP peak at 150° was enhanced by spiking with a
small amount of authentic AEP derivative. The putative AEP peak was
collected by preparative gas liquid chromatography and analyzed by mass
spectrometry. This analysis indicated the presence of AEP. Quantita-
tion of the glc chromatogram against authentic dimethyl 2-acetamido-

ethylphosphonate indicated that 0.1 mg AEP was present for each gram (wet weight) of rbc ghosts.

F. Presence of AEP in Human Heart and Skeletal Muscle

Human heart and skeletal muscle were separately homogenized in a Waring blender and hydrolyzed for 30 hr. in 500 ml 24% hydrobromic acid. The hydrolysates were filtered and defatted with three, 500 ml portions of chloroform. Heart hydrolysate (1.3 g) and skeletal muscle hydrolysate (1.1 g) were then derivatized by sequential acylation and methylation. These derivatized mixtures were subjected to preparative thin layer chromatography (see Section E-2, Chapter III) and these preparations were assayed for the AEP derivative by gas-liquid chromatography. Both of these human tissues were shown to contain AEP. In the case of human skeletal muscle, the putative AEP derivative peak was collected by preparative glc (six 14 µl injections) and identified as dimethyl 2-acetamidoethylphosphonate by mass spectrometry. The approximate amount of AEP present in human heart and skeletal muscle was determined by comparing the peak areas on the chromatograms from these fractions to the peak areas from known amounts of the authentic AEP derivative. Table IV shows the concentration of AEP (mg AEP/g wet tissue) in human heart and skeletal muscle as well as in human brain, liver and red blood cell membranes (ghosts).

Table IV - Concentration of AEP in Human Tissues.

Tissue	Concentration ($mg\ AEP/g$ wet tissue)
Brain	0.1 ; 0.2[*]
Liver	0.2
Skeletal Muscle	0.4
Heart	0.6
Red Blood Cell Membrane	0.1

[*] Two different brains

Chapter V - STUDIES ON THE BIOSYNTHESIS OF AEP IN A MAMMALIAN SYSTEM

A. Introduction

The previous chapter has given evidence for the occurrence of AEP in five human tissues. It is reasonable to suppose AEP to be generally distributed in human (and perhaps other mammalian) tissues. This wide-spread tissue distribution of AEP raises the interesting question of its origin in mammalian tissues. One possibility is that AEP is bio-synthesized by mammals. The experiments in this chapter were carried out to investigate this possibility. The radioactive precursors used (U-^{14}C-glucose, ^{32}P-orthophosphate and ^{14}C$_3$-pyruvate) were compounds which have been incorporated into AEP by Tetrahymena (see Section D of Introduction, Chapter I).

B. Presence of AEP in Rat Liver

Three male Sprague-Dawley rats were anesthetized by intraperitoneal injection of 25 mg Nembutal. Immediately after injection, their livers were dissected out (22.3 g) and homogenized in a Waring blender. To the homogenate, 125 ml of 24% hydrobromic acid was added and the mixture

was hydrolyzed for 24 hours under reflux. Distilled H_2O (50 ml) was added to the hydrolysate and the hydrolysate was filtered. The filtrate was extracted with five, 50 ml portions of chloroform.

A 7% aliquot (2.2 g) of the hydrolysate was subjected to ion-exchange chromatography on a 26.5 x 2 cm column of Dowex 50W-X4, H^+ form. Elution was carried out with 0.1N HCl and the first liter fraction was collected. Amino acid analysis performed on a 0.25% aliquot of this fraction indicated the probable presence of AEP (peak at 29 min.). A second 0.25% aliquot spiked with 6.2 μg authentic AEP was run on the amino acid analyzer. This enhanced the peak at 29 minutes. From these two analyses the concentration of AEP in rat liver was calculated to be 1.3 mg AEP per gram of rat liver (wet weight).

A second aliquot (10%) of the defatted rat liver hydrolysate was derivatized by sequential acetylation and methylation (Chapter III). This derivatized fraction was further purified by preparative tlc and then was subjected to gas liquid chromatography on a 6 ft x 1/4 in. 10% GE SE30 column (column A) at 150°, 160°, and 170° isothermally. At each temperature a peak was present in the derivatized rat liver hydrolysate with the retention time of authentic AEP derivative. Furthermore, "spiking" the derivatized rat liver hydrolysate with microgram amounts of the authentic AEP derivative enhanced the putative AEP peak without giving rise to any new peaks or shoulders on peaks. Quantitative analysis of these glc chromatograms (see Section C-3, Chapter III) indicated that the concentration of AEP in rat liver

was 0.9 mg AEP per gram of rat liver (wet weight). This value is similar to the value obtained by automatic amino acid analysis (1.3 mg/g rat liver).

C. In Vitro Studies

1. U-^{14}C-glucose Experiment.

Two male Sprague-Dawley rats (approximately 125 g/rat) were anesthetized by intraperitoneal injection of 25 mg Nembutal. The livers were immediately removed and put in ice-cold Krebs'-Ringer phosphate buffer. The chilled livers were sliced into approximately 0.5 mm thick slices with a Stadie-Riggs microtome (68). These weighed slices (3.5 g) were then placed in four flasks, each containing 2 ml of Krebs'-Ringer phosphate buffer and 10.4 μC of U-^{14}C-glucose per flask. The flasks were loosely covered with tin foil and incubated at 37° for 2 hours in a Dubonoff shaker. The incubation was terminated by addition of enough 48% hydrobromic acid to make the final mixture about 24% in hydrobromic acid. This mixture was hydrolyzed under reflux for 24 hr., filtered and diluted with 25 ml distilled H_2O. The diluted and cooled hydrolysate was then extracted (defatted) with three 60 ml portions of chloroform.

Twenty-five mg of authentic AEP was added to 43% of the defatted and charcoal-decolorized tissue hydrolysate. This sample was put on a Dowex 50W-X4, 200-400 mesh, H^+ form ion-exchange column (26.5 x 2 cm)

in about 3 ml of 0.1N HCl. Elution was carried out with 0.1N HCl and 100, 10 ml fractions were collected. Aliquots (300 μl) were taken from each fraction and neutralized with a pH 5.1 sodium acetate buffer. These aliquots were tested with ninhydrin and those which were positive (fractions 6,7,60-76) were subjected to thin layer chromatography on 160 micron cellulose plates against authentic AEP in two solvent systems: n-butanol: acetic acid: H_2O (3:1:1) and isopropanol: formic acid: H_2O (2:1:1). The tlc data indicated that AEP was probably in fractions 60-76. Therefore, these fractions were combined and a 0.2% aliquot was put in pH 2.2 citrate buffer and subjected to automatic amino acid analysis. This analysis gave a peak at 29 min., again indicating the probable presence of AEP in fractions 60-76.

The remaining portion of fractions 60-76 was evaporated on the rotary evaporator and yielded about 300 mg of residue. This residue was primarily an inorganic salt (probably NaCl) as determined by its very high melting point. Since AEP is soluble in concentrated HCl and NaCl is not, the residue was put in 1 ml of concentrated HCl and filtered through a fritted glass filter. This was done a second time and the concentrated HCl was evaporated under reduced pressure, yielding 40 mg of residue. This residue was dissolved in 1 ml of H_2O and hot absolute ethanol was added until turbidity resulted. A few drops of H_2O were added to eliminate the turbidity and the solution was allowed to cool slowly to room temperature. The resulting crystals (9 mg) were harvested and exhibited a melting point of 262-270° with decomposition (as compared to 262-274° for an authentic sample of AEP).

The crystals were dissolved in 1 ml distilled H_2O and a 10% aliquot was assayed for radioactivity in 10 ml of toluene: ethanol (2:1) by liquid scintillation counting (see Materials and Methods, Chapter II) for 73 minutes. The low activity (26 cpm/mg AEP) was comparable to background (22-25 cpm)[‡], indicating that the U-^{14}C-glucose had not been significantly incorporated (<0.001%) into AEP.

2. $^{14}C_3$-pyruvate and ^{32}P-orthophosphate Experiments.

Experiments similar to the one described above with U-^{14}C-glucose were carried out using $^{14}C_3$-pyruvate and ^{32}P-orthophosphate as precursors. Rat liver slices were used and a Krebs-bicarbonate buffer (see Materials and Methods) was used for the ^{32}P-orthophosphate incubation medium instead of the Krebs-Ringer phosphate buffer (which was used for the U-^{14}C-glucose and $^{14}C_3$-pyruvate incubation media). For each experiment the AEP was isolated as described, counted by liquid scintillation spectrometry and found not to be above background. Table V summarizes the conditions and results of the *in vitro* biosynthetic experiments.

D. In Vivo Study

U-^{14}C-glucose (27.5 µC) in 0.2 ml of sterile 0.9% saline was injected intraperitoneally into each of two male Sprague-Dawley rats

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[‡] At a 0.05 significance level this corresponds to a 4.5% error and thus the activity of the AEP (26±1.2 cpm/mg AEP) overlaps with background.

Table V - Summary of the conditions and results of the attempts to
biosynthesize AEP in an *in vitro* rat liver system (described
in the text).

	Precursors Used		
	U-^{14}C-glucose	^{14}C$_3$-Pyruvate	^{32}P-ortho-phosphate
Incubation Medium	Krebs-Ringer phosphate buffer	Krebs-Ringer phosphate buffer	Krebs-bicarbonate buffer
Incubation time	2 hr.	3 hr.	3 hr.
Incubation temperature	37°	37°	37°
Amount of liver slices	3.5 g	3.0 g	3.4 g
Radioactivity of precursor (specific activity)	41.6 μC (4.0 mC/mM)	59.5 μC (5.4 mC/mM)	0.4 mC (10 mC/ml)
Specific activity of isolated AEP (cpm/mg)	0*	0*	0*

* Activity of isolated AEP was not significantly greater than back-
ground. Specific activity was calculated as follows:

$$\frac{\text{cpm (AEP)} - \text{cpm (background)}}{\text{amount AEP (mg)}} = \text{specific activity}$$

(100-150 g). The rats were housed in a cage and given food (Purina Laboratory Chow) and water *ad libitum*. After 12 days the rats (approx. 200 g) were anesthetized by intraperitoneal injection of 25 mg Nembutal. The rats were skinned and the following tissues were dissected out: testes, peritoneum, heart, liver, thyroid, lungs, kidneys, skeletal muscles, and brain. These tissues (50 g) from both rats were combined and homogenized in 125 ml H_2O in a Waring blender. The homogenate was brought to about 24% hydrobromic acid and hydrolyzed under reflux for 28 hr. The hydrolysate was filtered and the water-soluble portion extracted with five, 300 ml portions of chloroform, yielding 33.1 g of defatted hydrolysate. To further concentrate the AEP, the hydrolysate was extracted with 400 ml acetone. This left 21.1 g of residue in the aqueous phase.

Chloroform-acetone-extracted hydrolysate (3.1 g) in 4.5 ml of 0.1N HCl (with 25 mg carrier AEP) was then put on a 2 x 26 cm Dowex 50W-X4, 200-400 mesh, H^+ form ion-exchange column. Elution was carried out with 0.1N HCl and 100, 10 ml fractions were collected (over approximately 5 hr.). Aliquots (300 µl) were taken from each fraction and neutralized with a pH 5.1 sodium acetate buffer. These aliquots were tested with ninhydrin and the positive fractions (6,7,50-63,86-100) were further investigated to see which fractions contained AEP. Automatic amino acid analysis of 1% aliquots of combined fractions 50-63 and combined fractions 86-100 yielded a peak at 28 min. for the former and at 49 min. for the latter. This indicated the probable presence of AEP in fractions 50-63. A 10% aliquot of fractions 50-63 was

evaporated to dryness, the residue dissolved in 100 μl H_2O and this solution radioassayed in 10 ml toluene: ethanol (2:1) with the appropriate scintillators. Counting for 10 min. revealed that the aliquot of fractions 50-63 was not significantly greater than background (8.6±1.0 cpm/mg AEP) at the 0.05% significance level (which corresponded to a 12% counting error).

Chapter VI – <u>GENERAL DISCUSSION</u>

In order to establish the presence of AEP in bovine brain by iso-
lation, Shimizu and coworkers (28) processed 28.5 kg of tissue. Several
other investigators (11,16,21) have relied on isolation and have pro-
cessed large (Kgm) quantities of tissues to demonstrate the presence of
AEP. Quite clearly, a prerequisite for an extensive investigation of
the occurrence of AEP in nature is the availability of a sensitive and
specific method for its determination. The present work has developed
a procedure which utilizes two highly sensitive instrumental techniques:
gas-liquid chromatography and mass spectrometry. Because each of these
techniques requires volatile samples for analysis, it is necessary to
derivatize tissue hydrolysates which are to be examined for the
presence of AEP. This is accomplished by carrying out sequential
acetylation and methylation reactions (Figure 5, Chapter III). Dimethyl
2-acetamidoethylphosphonate obtained in this way is a colorless, viscous
oil suitable for gas-liquid chromatographic and mass spectrometric
analysis. The mass spectrum of the AEP derivative (Figure 6, Chapter
III) exhibits an intense parent ion (m/e, 195) and characteristic
fragmentations which make identification straightforward. Very small
quantities (25-50 nanograms) of the volatile AEP derivative can be

detected by glc and small glc-isolated quantities (1-5 micrograms) can be unambiguously identified by mass spectrometry.

Many assays for naturally occurring compounds are based solely on chromatographic evidence and can lead to falsely positive results. The power of the present assay resides in the fact than an indication of probable presence of AEP (by amino acid analysis and glc) can be un- ambiguously confirmed through glc collection and mass spectrometric analysis of the putative AEP derivative. Since glc is a very sensitive chromatographic technique with high resolution, unambiguous characteri- zation by mass spectrometry is not necessary as a routine part of the AEP assay.* Co-chromatography with dimethyl 2-acetamidoethylphos- phonate by glc at different column temperatures and on columns with liquid phases of varying polarity provides good circumstantial evidence for the presence of AEP. However, analysis of samples by mass spectrom- etry is essential if unambiguous determination of AEP's presence is desired.

Since tissues and tissue extracts invariably yield complex mixtures on hydrolysis, methods had to be developed to purify and concentrate the AEP (when present) prior to glc analysis. Two alternative pro- cedures (for details see Section E, Chapter III) were employed, each having certain advantages. One procedure involves treatment of the chloroform-extracted hydrolysate on a Dowex 50W cation-exchange column.

* Many laboratories still do not have access to a mass spectrometer.

AEP and a few other acidic components of the hydrolysate emerge near the front when the column is eluted with 0.1N HCl. The majority of the hydrolysate's components are retained on the column at this low pH and the eluate is greatly enriched in AEP. This enriched fraction can then be derivatized and assayed for AEP. Alternatively, AEP in tissue hydrolysates can be concentrated using preparative thin layer chromatography of the derivatized hydrolysate on either alumina or silica gel plates. Although this method is not as effective as the Dowex treatment for purifying and concentrating AEP, it is less time consuming and easier to monitor. Whereas preparative tlc can be easily monitored with authentic dimethyl 2-acetamidoethylphosphonate as a marker on the edges of the thin layer plates, a more laborious testing of column fractions for AEP by amino acid analysis is usually necessary when the Dowex column purification is employed. In theory both procedures (Dowex 50W treatment followed by derivatization and preparative tlc) could be used on a complex hydrolysate containing little AEP. Since the two procedures rely on different chemical properties for their separations, the two procedures used sequentially should complement each other and thus yield much less complex fractions greatly enriched in AEP. The efficacy of the preparative tlc concentration of AEP was demonstrated when it allowed AEP to be detected in a polar lipid (methanol soluble) fraction of human brain in which AEP was not initially detected (31).

With the new assay adequately developed, a study of human tissues with diverse structures and functions was undertaken. Since previous

workers had shown AEP to be contained in phospholipid (polar lipid) fractions and in proteinaceous material (14,15), an initial hexane extraction was used (for brain and liver) in order to remove considerable amounts of material containing no AEP. However, contrary to expectations, analysis of this extract from brain indicated the presence of a significant amount of AEP (0.4 mg AEP/gram of fraction; AEP was not found in the corresponding hexane extract of lyophilized liver). Equally surprising, the concentration of AEP in the brain fraction (methanol soluble) thought to contain most of the phospholipids (72,73) was approximately four times less (0.1 mg AEP/g fraction) than in the hexane-soluble fraction. However, in the liver considerable AEP (0.6 mg/g fraction) was found in the methanol-soluble fraction and none (<50 µg AEP/g fraction) in the hexane-soluble fraction. The quite different distribution of AEP between polar and nonpolar lipid fractions in liver and brain is noteworthy but unexplainable at the present time. In each of the two fractionated tissues (brain and liver) AEP was found in highest concentration (1.0 mg AEP/g fraction) in the protein-rich fraction. A similar result was obtained in the study of beef brain (28). These results are also unexplainable at present but are consistent with AEP's postulated role as a constituent of proteins (7,14,15). Also consistent with this postulated function is the distribution of AEP which was found between different tissues. It seems that protein-rich tissues (77) contain the highest concentration of AEP. The overall concentration found in brain and liver (0.1 and 0.2 mg AEP/g wet tissue, respectively) is considerably less than the

concentration in skeletal muscle (0.4 mg AEP/g wet tissue) and heart
(0.6 mg AEP/g wet tissue). It is interesting and perhaps pertinent
that the tissue concentrations of AEP found in the present study are
of the same order of magnitude as concentrations of chemically similar
and metabolically important compounds such as phosphocreatine (in
tissues other than skeletal muscle) (78) and the common amino acids,
aspartic acid, alanine and glutamic acid (79,80).

Speculations have been made that AEP might be involved in phosphate
metabolism (30,47-53). In this regard, the observation that AEP and
its biosynthetic precursor in Tetrahymena (38-41), 2-amino-3-
phosphonopropionic acid (phosphonoalanine), undergo enzymatic trans-
aminations in the presence of amine acceptors to yield the correspond-
ing carbonyl derivatives (49,53) is potentially quite important (see
Figure 4, Chapter I). These carbonyl compounds are isoelectronic with
known biological (high-energy) metaphosphate transfer agents (e.g.,
phosphoenolypyruvate). The possibility that AEP (with the intermediacy
of the corresponding carbonyl derivative) is involved in highly specific
enzymatic transfers of high-energy phosphate is quite exciting.

As mentioned in the introduction, several investigators have con-
sidered that AEP may be involved as a structural component in membrane
phospholipids (36,44-46). Thompson has shown that phosphatidylamino-
ethylphosphonate is probably a membrane component in Tetrahymena (36)
and Rosenberg (45) and Chou and Scherbaum (46) have also given evidence
that AEP might be involved as a structural material of cell membranes.
A mammalian subcellular fraction or component has never been analyzed

for AEP even though AEP has been found in the cilia and mitochondria of Tetrahymena (44,81). Therefore, it was decided to purify a membraneous subcellular fraction from mammalian cells for AEP analysis. If AEP is present in a mammalian membraneous fraction, this would add to the circumstantial evidence for AEP's involvement in membrane phospholipids (36,44-46). The human red blood cell membrane (ghost) was chosen for study because it is relatively well characterized and easy to isolate (75,76). As anticipated, AEP was found to be present (0.1 mg AEP/g wet weight rbc ghosts) in the hydrolysate of rbc ghosts.

The present study has established the presence of AEP in four human tissues (brain, liver, heart and skeletal muscle) and one human sub-cellular fraction (rbc ghosts). It is reasonable to suppose AEP to be generally distributed in human (and perhaps other mammalian) tissues (32). However, it is difficult to assess the importance to mammalian biochemistry of the present finding. Other than the established presence of AEP in goat liver (27) and beef brain (28), there is very little published information concerning the occurrence of AEP in mammals. The authors making these reports (27,28) and later reviewers (3,4) have taken the view that the AEP in tissues of these ruminants very likely originated in metabolism of rumen microorganisms. The origin of AEP in human tissues is similarly uncertain; Quin (15) has noted that "one might ingest as much as 20 to 30 mg of 2-aminoethyl-phosphonic acid on eating four clams or eight mussels." The presence of AEP in beef and goat tissue indicate that dairy and meat products may be a source of AEP intake in humans. Regardless of whether AEP is

an endogenous or exogenous constituent of human tissues, it is important that an understanding be gained of its function in, or effect on, human biochemistry.

While it is evident that AEP could be ingested, the question of whether or not AEP arises biosynthetically in mammalian tissues has not been answered. Part of the present research has addressed itself to this question. Using the rat for these biosynthetic studies, *in vitro* liver slice experiments were done using radioactive precursors which had been incorporated into AEP in Tetrahymena (38-41). Rather long incubation times were used (2-3 hrs.) due to the only available information on AEP metabolism: the reported slow turnover of AEP in Tetrahymena (44,45,82). The rat liver slice system appeared to successfully metabolize the water-soluble precursors used (U-^{14}C-glucose, $^{14}C_3$-pyruvate and ^{32}P-orthophosphate). This was indicated when considerable radioactivity was extracted by chloroform from the rat liver slice hydrolysate.* Despite the use of large amounts of radioactivity in the precursors (41-400 µC) no significant incorporation of radioactivity (<0.001%) into AEP could be demonstrated. In each experiment the AEP was isolated by ion-exchange chromatography but liquid scintillation counting indicated that the AEP was no more radioactive than background.

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* Approximately 40% of the radioactivity accounted for was in the chloroform extract.

One *in vivo* biosynthetic study was also conducted with rats. $U-^{14}C$-glucose was used as the precursor and the rats were allowed to metabolize the precursor for twelve days. However, liquid scintillation spectrometry indicated that the fraction containing the AEP from the rats' tissues was not above background. It appears that AEP was not biosynthesized (<0.001% incorporation of label) from the particular precursors used and by the *in vitro* and *in vivo* rat systems utilized in the experiments reported in Chapter V.

SUMMARY AND CONCLUSIONS

The research in this thesis was primarily concerned with the development of an assay for AEP which could be utilized for investigating the occurrence, distribution and concentration of AEP in human tissues. The following conclusions and interpretations can be drawn from the studies:

A. Conclusions:

1. A new sensitive, specific and unambiguous assay is now available for the detection of AEP in biological materials.

2. AEP has been found to be present in rather large concentrations (0.1-1.0 mg per gram of tissue or tissue fraction) in all the human tissues investigated (brain, liver, heart, skeletal muscle and blood).

3. Protein-rich tissues and the proteinaceous fraction (hexane-methanol insoluble) of brain and liver tissues contain the largest concentration of AEP.

4. AEP is present in a subcellular component of a mammalian cell, the cell membrane (ghost) of the human erythrocyte.

5. Radioactivity from U-^{14}C-glucose, ^{14}C$_3$-pyruvate and ^{32}P-orthophosphate was not significantly incorporated (<0.001%) into AEP under the conditions employed in the *in vitro* (rat liver slices) and *in vivo* (rat) biosynthetic experiments.

B. Interpretations:

1. AEP is widely distributed in human (and perhaps other mammalian) tissues.

2. The findings in this thesis are consistent with, but not proof of, a structural role for AEP in proteins and/or cellular membranes.

3. AEP does not appear to be biosynthesized by the rat (*in vitro* or *in vivo*).

4. Although the studies reported in this thesis have not addressed themselves directly to the question of AEP's function, they should be valuable in stimulating and facilitating an answer to the important question of AEP's function in, or effect on, mammalian systems.

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Occurrence of 2-Aminoethylphosphonic Acid
in Human Brain

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Occurrence of 2-Aminoethylphosphonic Acid in Human Brain[*]

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ABSTRACT: The occurrence of 2-aminoethylphosphonic acid (AEP) in acid hydrolysates of nonpolar lipid (hexane soluble) and proteinaceous residue (hexane–methanol insoluble) fractions of human brain tissue has been unambiguously established. This was accomplished using a new, highly sensitive technique for analysis of AEP in biological materials. This method involves acylation and esterification of hydrolysates of tissue extracts, detection and isolation by gas–liquid chromatography, and unambiguous characterization by mass spectrometry.

The presence of 2-aminoethylphosphonic acid (**1**, R = H) in hydrolysates of human brain has been established using a new, highly sensitive and unambiguous technique. The occurrence in human brain of AEP[1] which possesses the biologically unusual carbon–phosphorus bond, suggests that it and perhaps other, closely related, naturally occurring phosphonic acids (Kittredge and Roberts, 1969; Quin, 1967), may have important, but as yet undiscovered, functions in human biochemistry.

Following its initial isolation from ciliated protozoa of sheep rumen (Horiguchi and Kandatsu, 1959), AEP and four closely related compounds have been isolated from a variety of natural sources—principally marine invertebrates and microorganisms (Kittredge and Roberts, 1969; Quin, 1967). Surprisingly, no systematic study of the occurrence of aminoethylphosphonic acids in higher animals has been made, although the presence of AEP in goat liver (Kandatsu and Horiguchi, 1965) and bovine brain (Shimizu et al., 1965) and of trimethylaminoethylphosphonate (**2**) in human aorta (Bishop, 1968) has been reported.

[1] Abbreviation used is: AEP, 2-aminoethylphosphonic acid.
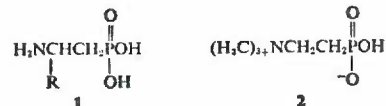
Experimental Section

General Comments. Infrared spectra were determined with a Perkin-Elmer 337 grating spectrophotometer. Nuclear magnetic resonance spectra were determined with a Varian HA-100 spectrometer. Mass spectra were obtained with a CEC Model 21-110B mass spectrometer at 100° probe temperature and 70 eV. Amino acid analyses were done on a Beckman 120C Analyzer using the method of Moore *et al.* (1958). Melting points were taken on a microscope hot stage and are uncorrected. Gas–liquid chromatography was carried out using a F & M Model 810 gas chromatograph with a flame ionization detector. A prepoured glass column (6 ft $\times$ 0.25 in.) with 10% GE SE30 on a A-W Chromosorb G (Applied Science) was used under the following conditions: H_2 pressure of 18 psig; air pressure of 20 psig; N_2 flow rate of 60 cc/min; injector temperature of 220°; flame ionization detector temperature of 315°. Elemental analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Ion-exchange chromatography was accomplished using a 2 $\times$ 27 cm column of Dowex 1-X8, 200–400 mesh, acetate form.

2-Acetamidoethylphosphonic Acid (3). To 42.1 mg of 2-aminoethylphosphonic acid[2] (Kosolapoff, 1947) was added 8 ml of acetic acid–acetic anhydride (1:1, v/v). The resulting mixture was stirred at room temperature until complete solution was obtained (approximately 2 hr). The solution was then filtered and the volatile reagents were removed. The resulting residue was crystallized from methanol to yield 31.6 mg (75%) of 2-acetamidoethylphosphonic acid (3); mp 172–180°; nuclear magnetic resonance (given in τ values throughout) spectra (Me$_2$SO-d_6), 2.19, broad (NH); 6.82, multiplet (N-CH_2); 8.24, singlet (CH_3C=O); 8.32, multiplet (P-CH_2); infrared spectrum (KBr) 1590 cm^{-1} (C=O).

Anal. Calcd for $C_4H_{10}NO_4P \cdot 0.5H_2O$: C, 27.3; H, 6.26; N, 7.96. Found: C, 27.5; H, 6.27; N, 7.90.

Dimethyl-2-acetamidoethylphosphonate (4). To a solution of 30.0 mg of 2-acetamidoethylphosphonic acid (3) in 5 ml of methanol was added excess diazomethane in ether. The solvents were evaporated using a stream of nitrogen to yield a viscous liquid, 25.8 mg (86%) of dimethyl-2-acetamidoethylphosphonate (4). The nuclear magnetic resonance spectra showed (CDCl$_3$), 3.44, broad (NH); 6.24, doublet (6 H, OCH_3); 6.50, multiplet (N-CH_2); 7.98 multiplet (P-CH_2); 8.20, singlet (CH_3C=O); infrared spectrum (smear), 1655 cm^{-1} (C=O); mass spectrum: see Figure 1.

Anal. Calcd for $C_6H_{14}NO_4P$: C, 36.9; H, 7.19; N, 7.18. Found: C, 36.9; H, 7.37; N, 7.08.

Model Studies. A mixture of 2.0 mg of AEP and 30.0 mg of each of 5 amino acids (Glu, Val, Phe, Thr, and Asp) in 6 ml of acetic acid–acetic anhydride (1:1) was stirred for 3 hr. The solvent was evaporated, the residue was dissolved in 50 ml of methanol and a 5-ml aliquot was treated with excess diazomethane in ether. The resulting mixture was evaporated, the residue was dissolved in 1 ml of chloroform and subjected to gas–liquid chromatographic analysis using the gas–liquid chromatographic column and conditions described under General Comments. The AEP derivative, 4, was readily separated from the amino acid derivatives at isothermal column temperatures of 170, 180, or 200° exhibiting retention
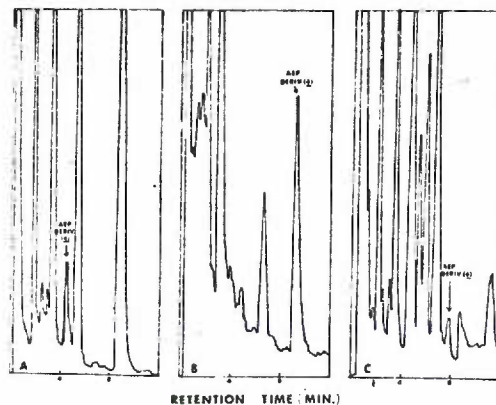
FIGURE 1: Typical gas–liquid chromatograms of complex mixtures showing separation of AEP derivative 4 obtained using instrumentation and conditions as described (general comments). (A) Mixture of derivatized AEP and amino acids (see model studies); column 200° isothermal; AEP derivative retention time = 4.4 min. (B) Derivatized hydrolysate of nonpolar lipid (hexane soluble) fraction; column 170° isothermal; AEP derivative retention time = 9.6 min. (C) Derivatized hydrolysate of proteinaceous residue (hexane–methanol insoluble) fraction; column 180° isothermal; AEP derivative retention time = 7.4 min.
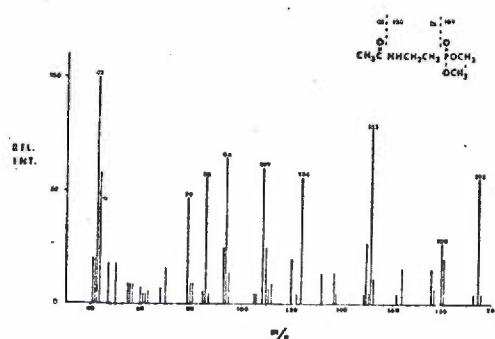
times of 9.6, 7.4, and 4.4 min, respectively (see Figure 1A). Various programmed column temperature regimes were equally effective or, in some cases superior. Comparison of these gas–liquid chromatographic results with those obtained using standard solutions of 4 indicated that essentially quantitative conversion of AEP to the volatile derivative 4 had been achieved.

Fractionation of Brain Tissue. An adult human brain[3] was homogenized in a Waring blender and subsequently lyophilized. The lyophilized tissue (36.7 g) was extracted with 3.5 l. of redistilled hexane by shaking mechanically overnight. The hexane extract was removed, dried over sodium sulfate, and the solvent was removed to yield 12.5 g of residue (nonpolar lipids). The hexane-insoluble residue was extracted using 3 l. of methanol for 12 hr. The methanol extract was separated from the hexane-methanol-insoluble material (14.3 g of proteinaceous residue). Removal of solvent from the methanol extract produced 7.8 g of polar lipids.

Analysis of Nonpolar Lipid (Hexane Soluble) Fraction for AEP. A portion (8 g) of the hexane-soluble material was hydrolyzed by heating for 19 hr in 500 ml of 24% hydrobromic acid under reflux. The cooled hydrolysate was diluted with 500 ml of water and extracted with 2.5 l. of chloroform to remove lipids. The aqueous portion of the hydrolysate was evaporated under reduced pressure to yield 3 g of ninhydrin-positive residue which was fractionated on a Dowex 1-X8, 200–400 mesh column in the acetate form. Elution was carried out with distilled water; the compound of interest was eluted

FIGURE 2: Mass spectrum of dimethyl 2-acetamidoethylphosphonate (4).

TABLE I: Concentration of 2-Aminoethylphosphonic Acid (AEP) in Human Brain Fractions.

Fraction	Concn (mg of AEP/g of Fraction)
Nonpolar lipids, 12.5 g (hexane soluble)	~0.4
Polar lipids, 7.8 g (methanol soluble)	Not detected
Proteinaceous residue, 14.3 g (hexane–methanol insoluble)	~1

in the first 50 ml (fraction I). This fraction was ninhydrin positive and contained a compound which was indistinguishable from authentic AEP by descending paper chromatography (Whatman No. 1) in the following solvent systems: (a) 1-butanol–acetic acid–H_2O (4:1:4, v/v), R_F 0.38; (b) isopropyl alcohol–formic acid–H_2O (8:1:1, v/v), R_F 0.11. Also cochromatography by ascending paper chromatography was exhibited in the following solvent systems: (c) methanol–pyridine–H_2O (17:1:5, v/v), R_F 0.43; (d) pyridine–H_2O (65:55, v/v), R_F 0.42. An aliquot of fraction I gave a negative test for P_i (Chen et al., 1956); a second aliquot, following oxidation with 70% perchloric acid (Hirata and Appleman, 1959), gave a positive phosphate test indicating that fraction I contained one or more compounds possessing a C–P bond.

A third aliquot was evaporated, brought up to 1 ml with pH 2.2 citrate buffer and examined using the automatic amino acid anlyzer. Using a 55-cm column a band was eluted (28 min) which corresponded to that of authentic AEP (Kittredge and Hughes, 1964; Chou and Scherbaum, 1966; Hori et al., 1967).

The remainder of fraction I was derivatized in the manner described (see model studies) and the resulting mixture was examined by gas–liquid chromatography. A compound was observed which exhibited retention times identical with those of synthetic dimethyl 2-acetamidoethylphosphonate (4) using six different temperature regimes (e.g., see Figure 1B). This compound was isolated by preparative gas–liquid chromatography using a column temperature of 170° (eight 10-μl injections). A mass spectrum of the collected material exhibited a parent ion, m/e 195.0650 (calcd for 4, 195.0654) and fragment ions identical with those observed in a spectrum of synthetic 4 (Figure 2).
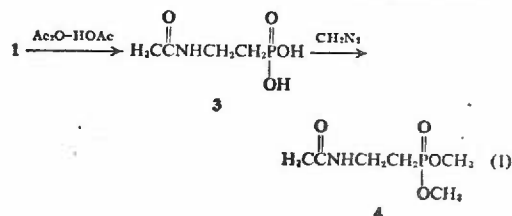
Analysis of Polar Lipid (Methanol Soluble) and Proteinaceous Residue (Hexane–Methanol Insoluble) Fractions for AEP. Samples (2 g) of each of the fractions were hydrolyzed using 24% hydrobromic acid and extracted with chloroform as described for the nonpolar lipid (hexane soluble) fraction. The respective aqueous phases were evaporated to dryness and derivatized for gas–liquid chromatographic analysis as described. A peak corresponding to 4 was observed in the chromatogram for the proteinaceous residue (Figure 1C). This material was collected from the gas–liquid chromatographic
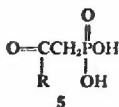
column (thirteen 10-μl injections) and a mass spectrum was obtained which exhibited a molecular ion and a fragment pattern identical with that shown in Figure 2. Gas–liquid chromatographic analysis of the derivatized mixture obtained from the polar lipid (methanol soluble) fraction hydrolysate exhibited no peak with a retention time assignable to 4.

Concentration of AEP in Fractions. An estimate of the concentrations of the AEP derivative 4 in the nonpolar lipid and proteinaceous residue fractions was made by comparing the areas of the appropriate gas chromatographic peaks with peak areas obtained using known amounts of authentic 4. The assumption was made that complete conversion of AEP into 4 had been achieved (see Model Studies). The results, corrected to indicate concentration of AEP rather than of 4, are shown in Table I.

Results and Discussion

In order to establish the presence of AEP in bovine brain by isolation, Shimizu and coworkers (1965) processed 28.5 kg of tissue. Quite clearly, a prerequisite for an extensive investigation of the occurrence of AEP in nature is the availability of a sensitive method for its determination. We have developed a new procedure which utilizes two highly sensitive instrumental techniques—gas–liquid chromatography and mass spectrometry. Since each of these techniques requires volatile samples for analysis, it is necessary to derivatize mixtures to be examined for the presence of AEP. We accomplished this by carrying out sequential acylation and methylation reactions (eq 1). Dimethyl 2-acetamidoethylphosphonate (4) obtained in this way is a colorless viscous oil suitable for gas–liquid chromatographic and mass spectrometric analysis.[4] The mass spectrum of 4 (Figure 2) exhibits an intense parent ion and characteristic fragmentations which make identification straightforward.

[4] Recently Karlsson (1967) has reported the use of a trimethylsilyl derivative of AEP in a similar procedure.

$$O=CCH_2POH$$

with R below the first carbon and OH below the P, and O double-bonded above the P.

5

To assess the suitability of the technique for detection of AEP in complex mixtures, a model study was carried out. When AEP (<1%) in a mixture of amino acids was treated as indicated (eq 1) conversion into 4 was essentially complete and the resulting mixture was readily resolved by gas–liquid chromatography (Figure 1A).

With our method adequately developed we undertook a study of human brain tissue. Since previous workers had shown AEP to be contained in phospholipid (polar lipid) fractions and in proteinaceous material (Quin, 1964, 1965), we elected to use an initial hexane extraction which was expected to remove considerable amounts of material containing no AEP. Contrary to our expectations, however, analysis of this extract showed a significant amount of AEP to be present. Equally surprising AEP was not detected in the methanol-soluble fraction thought to contain most of the phospholipids (Ansell and Hawthorne, 1964; Entenman, 1957). The fraction richest in AEP (Table I), however, was the proteinaceous residue in agreement with the results for beef brain (Shimizu et al., 1965).

The discovery that AEP is present in both lipid and proteinaceous fractions of human brain, as well as in goat liver (Kandatsu and Horiguchi, 1965) and beef brain (Shimizu et al., 1965) suggests that a possible role for AEP in mammalian biochemistry should be considered. In this regard, the observation that AEP and its biosynthetic precursor in Tetrahymena (Trebst and Geike, 1967; Warren, 1968; Liang and Rosenberg, 1968; Horiguchi et al., 1968), 2-amino-3-phosphonopropionic acid (1, R = COOH) undergo enzymatic transaminations in the presence of amine acceptors to yield the corresponding carbonyl derivatives 5 (R = H, COOH) (Roberts et al., 1968; La Nauze and Rosenberg, 1968) is potentially very important. The carbonyl compounds 5 (R = H, COOH) are isoelectronic with known biological (high-energy) meta-phosphate-transfer agents (e.g., phosphoenolpyruvate). The possibility that AEP (with the intermediacy of the corresponding carbonyl derivative 5 (R = H)) is involved in highly specific enzymatic transfers of high-energy phosphate is an intriguing one.

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BBA Report

BBA 21309

2-Aminoethylphosphonic acid: Distribution in human tissues

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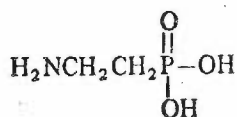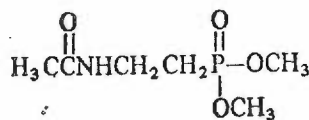[b] Oregon Graduate Center, Beaverton, Oreg. 97005 (U.S.A.)

SUMMARY

2-Aminoethylphosphonic acid (AEP) was identified in four human tissues. Its concentration was higher in heart and skeletal muscle than in liver or brain. The distribution of AEP between polar and nonpolar fractions was examined and differed considerably. AEP was found in highest concentration in the protein-rich fraction.

2-Aminoethylphosphonic acid (AEP, I), which is biosynthetically unique in that it contains a carbon–phosphorus bond, has been found in each of four human tissues examined – brain[1], liver, heart and skeletal muscle. Earlier investigations of the occurrence of AEP in natural materials have been directed primarily toward marine invertebrates and other lower animals[2–4] although the presence of AEP in goat liver[5] and beef brain[6] has been established. The present results, which suggest that AEP is generally distributed in human tissues and in surprisingly high concentrations, indicate a need for an investigation of the role of AEP in human and other mammalian biochemistry.

$$H_2NCH_2CH_2\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-OH \qquad\qquad H_3C\overset{\overset{O}{\|}}{C}NHCH_2CH_2\overset{\overset{O}{\|}}{\underset{\underset{OCH_3}{|}}{P}}-OCH_3$$

I   II

Analyses of tissues for AEP were accomplished using a facile and sensitive technique[1] which involves, initially, sequential acetylation (acetic anhydride–acetic acid) and methylation (diazomethane) of residues obtained by removal of solvent from acid hydrolysates of tissues or tissue extracts. In this way AEP (if present in a tissue sample) is converted quantitatively to a nonpolar derivative, dimethyl acetamidoethylphosphonate (II).

Abbreviation: AEP, aminoethylphosphonic acid.

Following this preliminary derivatization each sample was subjected to preparative thin-layer chromatography (0.5-mm alumina plates developed twice with acetone) using an authentic sample of dimethyl acetamidoethylphosphonate (II) as a marker (R_F approx. 0.2). The materials obtained by elution of the appropriate zones with chloroform were analyzed for the presence of the AEP derivative (II) by gas–liquid chromatography.

The use of a preliminary thin-layer chromatographic separation, which was not included in the method previously described[1], considerably improves the sensitivity of the gas–liquid chromatographic analysis. Using this step we were able to detect the AEP derivative II in preparations from the methanol-soluble (polar lipid) fraction of human brain which was not possible using the previous technique in which this step was omitted. The identity of II obtained by gas–liquid chromatography from tissue preparations was confirmed by mass spectrometry.

In Table I are presented the data obtained in this study. AEP was shown to be present in each of the four tissues studied. The brain, liver and skeletal muscle were taken from a 38-year-old caucasian male who died as a result of a crushing chest injury. Post-mortem examination showed no evidence of systemic disease. These tissues were removed, frozen and stored (3 years) until used. The heart was from a 27-year-old caucasian female who died of a heroin overdose. This tissue was removed directly after autopsy and immediately processed as described in the text. While the initial demonstration of the occurrence of AEP in human tissue was achieved using brain fractions[*], the concentration found in this tissue (0.1 mg/g wet tissue) is considerably less than the concentration in heart (0.6 mg/g wet tissue) or skeletal muscle (0.4 mg/g wet tissue). The quite different distribution of AEP between polar and nonpolar lipid fractions in liver and brain is noteworthy. Also, in each of the two fractionated tissues (brain and liver) AEP was found

TABLE I

DISTRIBUTION AND CONCENTRATION OF AEP IN HUMAN TISSUES

Tissue	Concentration of AEP[*]	
	(mg/g wet tissue)	(mg/g fraction)
Brain	0.1	
Hexane soluble (nonpolar lipids)		0.4[**]
Methanol soluble (polar lipids)		0.1[**]
Hexane–methanol insoluble (proteinaceous residue)		1.0[**]
Liver	0.2	
Hexane soluble (nonpolar lipids)		Not detected
Methanol soluble (polar lipids)		0.6
Hexane–methanol insoluble (proteinaceous residue)		1.0
Heart	0.6	
Skeletal muscle	0.4[**]	

[*]Obtained by gas–liquid chromatographic analysis, see. ref. 1.
[**]Identity of gas–liquid chromatographic peak confirmed by mass spectrometry (ref. 1).

[*]Subsequently, AEP has been shown to be present in a second human brain.

in highest concentration in the protein-rich fraction. A similar result was obtained in the study of beef brain[6]. It is interesting and perhaps pertinent that tissue concentrations of AEP found in the present study are of the same order of magnitude as concentrations of chemically similar and metabolically important compounds such as phosphocreatine (in tissues other than skeletal muscle)[7] and the common amino acids, aspartic acid, alanine and glutamic acid[8,9].

It is difficult to assess the importance to mammalian biochemistry of the present results. Other than the established presence of AEP in goat liver[5] and beef brain[6], there is very little published information concerning the occurrence of AEP in mammals. The authors making these reports[5,6] and later reviewers[2-4] have taken the view that the AEP in tissues of these ruminants very likely originated in metabolism of rumen microorganisms. The origin of AEP in human tissues is similarly uncertain; Quin[10] has noted that "one might ingest as much as 20 to 30 mg of 2-aminoethylphosphonic acid on eating four clams or eight mussels". The presence of AEP in beef and goat tissue indicate that dairy and meat products may be a source of AEP intake in humans. While it is evident that AEP is ingested, the question of whether or not AEP arises biosynthetically in mammalian tissue is open.

The present study has established the presence of AEP in four human tissues; it is reasonable to suppose AEP to be generally distributed in human (and perhaps other mammalian) tissues. Regardless of whether AEP is an endogenous or exogenous constituent of human tissue, it is important that an understanding be gained of its function in, or effect on, human biochemistry.

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