Synthesis and Biological Evaluation of an N^6-Diethylphospholysyl Peptide—A Model Compound with a Covalently Attached Metabolite of an Organophosphate Pesticide†

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A model peptide containing N^6-diethylphospholysine on an internal position, Ac-Ala-Lys(PO_3Et_2)-Val-OEt, and two nonphosphorylated peptides, Ac-Ala-Lys-Val-OEt and Ac-Tyr-Lys-Val-OEt, were synthesized in high yields and fully characterized by physicochemical constants, HPLC, and NMR and mass spectra. A rat growth study was conducted to determine bioavailability of lysine in the three peptides. A wheat gluten (20%) basal diet, adequate in all nutrients except lysine (0.26%), was supplemented with 0.1% crystalline lysine. Lysine bioavailability was calculated by comparing growth of rats fed the peptide diets with that of rats fed basal plus 0.1% crystalline lysine diet. Values for bioavailability of lysine in Ac-Ala-Lys(PO_3Et_2)-Val-OEt, Ac-Ala-Lys-Val-OEt, and Ac-Tyr-Lys-Val-OEt were 0, 79, and 95%, respectively.

Keywords: *Phosphorylation; protein-bound lysine; N^6-diethylphospholysine; organophosphate pesticides; model peptides; coupling; ^1H, ^{13}C, ^{31}P NMR spectra*

INTRODUCTION

Phosphorylation of proteins by organophosphate pesticides consists of covalent attachment of a dialkyl phospho group to a reactive functionality in a protein chain. The mechanism and biological consequences of phosphorylation of a serine residue in the active center of the enzyme cholinesterase are very well understood (Matsumura, 1975; Eto, 1974a). The intrinsically reactive nature of organophosphate pesticides renders them liable to a number of other reactions with tissue constituents. For example, it is well-known that organophosphate pesticides inhibit other tissue esterases, which are not essential to physiological processes, presumably by phosphorylating serine in their active centers (Environmental Health Criteria 63, 1986). Phosphorylation of other protein-bound amino acids, especially those that have more nucleophilic functionalities in their side chains such as lysine, could also occur.

Phosphorylation of proteins in plants by organophosphorus compounds can be expected to proceed in a similar manner as that in mammalian systems. Phosphorylation of amino acids in proteins utilized for food and feed purposes could adversely affect their bioavailability and their biological activity. It is not known whether phosphorylation of the terminal amino group of protein-bound lysine by organophosphorus compounds affects its bioavailability. We synthesized a large amount of model peptide containing N^6-diethylphospholysine on the internal position, Ac-Ala-Lys(PO_3Et_2)-Val-OEt (**2**) (Figure 1B; Table 1), and two nonphosphorylated control peptides, Ac-Ala-Lys-Val-OEt (**1**) and Ac-Tyr-Lys-Val-OEt (**3**) (Table 1), and determined their physicochemical constants and the bioavailability of lysine from them in weanling rats. This paper presents the results of these investigations.

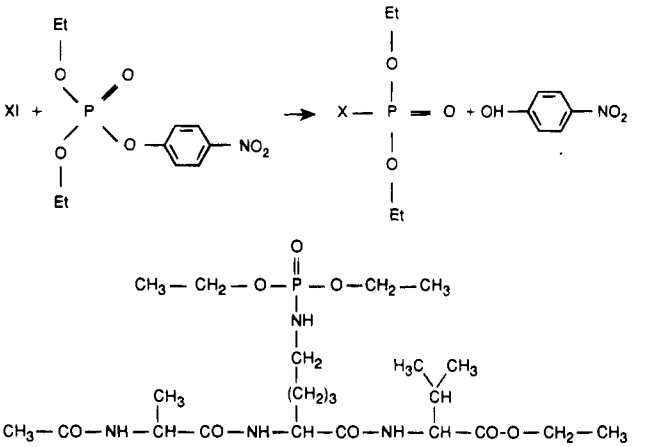

Figure 1. (A, top) Reaction of paraoxon with nucleophile X. (B, bottom) Tripeptide **2**.

MATERIALS AND METHODS

Preparation of Peptides. *General.* Boc-Lys(Z), Val-OEt·HCl, Boc-Ala, and Boc-Tyr(Bzl) were purchased from Sigma Chemical Co., St. Louis, MO. Diethyl phosphochloride was obtained from Aldrich Chemical Co., Milwaukee, WI.

Coupling Reactions. Ac-Ala-Lys(Z)-Val-OEt, the starting material for the synthesis of test compounds Ac-Ala-Lys-Val-OEt·HCl (**1**) and Ac-Ala-Lys(PO_3Et_2)-Val-OEt (**2**), was prepared using Boc-mode synthesis in solution with EDC as coupling reagent (Bodanszky et al., 1976). Reactions were done in DCM, and 50% TFA in DCM was used to remove the Boc-amino protecting groups in each step (Paquet et al., 1985). The coupling reactions were worked up by successive washing with aqueous solutions of sodium bicarbonate (10%, three times) and citric acid (10%, three times) and with water until neutral. Solutions were dried over sodium sulfate, and solvents were evaporated on a rotary evaporator. Purification was done by repeated precipitation (two times) from diethyl ether; purity was 99.7% (HPLC in acetonitrile–water 65:35) (Table 1).

Ac-Tyr(Bzl)-Lys(Z)-Val-OEt, the starting material for the test peptide Ac-Tyr-Lys-Val-OEt (**3**), was obtained similarly to Ac-Ala-Lys(Z)-Val-OEt with the exception that Boc-Tyr(Bzl) was incorporated into the chain instead of Boc-Ala. Precipita-

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Table 1. Characteristics of the Peptides[a]

compound	mp (°C)	$[\alpha]^{22}_D$[b]	formula	mass spec[c] (EI)
Ac-Lys(Z)-Val-OEt[d]	amorph	−49.4° (*c* 0.70)	$C_{26}H_{40}N_4O_7$	
Ac-Ala-Lys-Val-OEt·HCl (1)	amorph	−59° (*c* 0.50)	$C_{18}H_{35}N_4O_5Cl$	387 (7, M − HCl), 300 (10, CO-Lys-Val-OEt), 129 [30, $CH(CH(CH_3)_2COOCH_2CH_3)$], 84 [100, $(COCHCH(CH_3)_2]$, 56 [20, $CHCH(CH_3)_2$]
Ac-Ala-Lys(PO_3Et_2)-Val-OEt (2)[e]	134−135	−48°[f] (*c* 0.73)	$C_{22}H_{43}N_4PO_8$	522 (4 M), 477 [4, M − 45(OEt)], 378 (33, 522 − 144(NH-Val-OEt)], 377 [40, 522-100(Ac-Ala)-45-(OEt)], 220 [100, $(C(CH_2)_3CH_2NHPO(OEt)_2]$, 84 [48, $(COCHCH(CH_3)_2]$, 56(5)
Ac-Tyr(Bz)-Lys(Z)-Val-OEt[d]	amorph	−3°[f] (*c* 0.88)	$C_{39}H_{50}N_4O_8$	
Ac-Tyr-Lys-Val-OEt·HCl (3)	amorph	−2.5° (*c* 1.94)	$C_{24}H_{39}N_4O_6Cl$	478 (7, M − HCl), 300 (5, CO-Lys-Val-OEt), 206 (5, Ac-Tyr-CO), 178 [8, 206-28(CO)], 84 [100, $COCHCH(CH_3)_2]$, 56 (17)

[a] ^{1}H-NMR spectra of all compounds (CH_3OD) were in accord with the structure of the compounds. [b] In methanol unless otherwise noted. [c] *m/z* (relative intensity); major fragments of compounds **1**−**3** shown only. [d] EI mass spectra gave fragments which correlated with the structure of the products. [e] ^{31}P NMR (CH_3OD) showed singlet at +11.66 ppm. [f] In $CDCl_3$.

tion (two times) from diethyl ether gave the product of 98.5% purity (HPLC in acetonitrile−water 70:30) (Table 1).

Melting points were used to prove the purity of the products and were determined on an electrothermal apparatus by a capillary method. Values are shown in Table 1.

Optical rotations were determined to characterize the compounds using a Perkin-Elmer Model 241 polarimeter. Results are shown in Table 1.

Mass spectroscopy was used to prove the structure of the products. The spectra were recorded on a Finnigan MAT 4500 mass spectrometer using INCOS as data system. Samples were analyzed by direct insertion probe, under standard electron impact (EI) and methane chemical ionization (CI) conditions. Both techniques gave mass fragments confirming the structure of the products. Major fragments of EI spectra of the test compounds **1**−**3** are shown in Table 1, and Figure 3 depicts the whole spectrum of **2**.

NMR Spectroscopy. ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra were obtained at 500.13, 125.7, and 202.5 MHz, respectively, on a Bruker AM 500 NMR spectrometer. Chemical shifts were referenced to deuteriochloroform ($CDCl_3$) at 77.0 (^{13}C) and 7.24 ppm (^{1}H) and are reported relative to tetramethylsilane. The ^{31}P-NMR spectrum is referenced to an external standard of 85% phosphoric acid. The position of the ethyl ester of the phospho moiety was confirmed by the measurement of carbon−phosphorus couplings (J_{C-P}) in the ^{13}C-NMR spectra.

Analytical HPLC. These analyses were carried out to prove the purity of the products with a Varian 5020 liquid chromatograph on a 10-μm Bondapack C_{18} column, using UV absorbency detection at 208 nm. Elution solvents were mixtures of acetonitrile with water containing 0.1% phosphoric acid.

Synthesis of Ac-Ala-Lys-Val-OEt·HCl (**1**). To a solution of Ac-Ala-Lys(Z)-Val-OEt (27.06 g, 52 mmol) in 80% ethanol and 1 N HCl (52 mL, 1 equiv) was added palladium on charcoal catalyst (10%, 2.72 g), and the reaction was stirred in a hydrogen atmosphere overnight. The catalyst was filtered off and the filtrate evaporated to dryness, giving 22.0 g (quantitative yield) of the amorphous product **1**. Characteristics are indicated in Table 1.

Synthesis of Ac-Ala-Lys(PO_3Et_2)-Val-OEt (**2**). To a stirred and cooled (5 °C) suspension of **1** (11 g, 26.0 mmol) in acetonitrile (400 mL) was added triethylamine (2.89 g, 28.6 mmol), and stirring was continued until all of it was dissolved. The solution was cooled with ice, and diethyl phosphochloride (4.93 g, 28.6 mmol) and triethylamine (2.89 g, 28.6 mmol) were added. Stirring was continued for an additional 4 h. The mixture was evaporated on a rotary evaporator to a small volume, and the residue was dissolved in a mixture of chloroform with isopropyl alcohol (3:2, 300 mL), washed with water (three times, one-third of the volume), and dried over sodium sulfate. The solvents were evaporated, and the crude product was crystallized from ether, giving 12.24 g (90%) of the title compound. Purity was 98.8% (HPLC in acetonitrile−water 35:65). Characteristics are presented in Table 1.

Table 2. Composition of Basal Diet (As Is Basis)

ingredient	amount (g/kg)	ingredient	amount (g/kg)
wheat gluten[a]	200.00	choline bitartrate	2.00
corn oil (Mazola)	100.00	essential amino acid mixture[d]	6.50
cellulose (Alphacel)	50.00	sucrose	200.00
AIN-76 mineral mixture[b]	35.00	cornstarch	396.50
AIN-76A vitamin mixture[c]	10.00		

[a] Wheat gluten contained 87.69% crude protein (N × 6.25). [b] American Institute of Nutrition (1977). [c] American Institute of Nutrition (1980). [d] Essential amino acid mixture provided the following amino acids (g/kg of amino acid mixture): His·HCl·H_2O, 307.7; Met, 307.7; Thr, 230.8; Trp, 76.9; Arg, 76.9.

Synthesis of Ac-Tyr-Lys-Val-OEt·HCl (**3**). To a solution of Ac-Tyr(Bzl)-Lys(Z)-Val-OEt (5.0 g, 6.69 mmol) in a mixture of TFA−AcOH (70 mL) was added Pd catalyst (0.750 g, 15% on charcoal), and the mixture was stirred in a hydrogen atmosphere overnight. The catalyst was filtered off and the filtrate evaporated to dryness and dried in a vacuum to give 3.89 g (97%) of amorphous Ac-Tyr-Lys-Val-OEt·TFA. The TFA salt (7.5 g 12.65 mmol) was dissolved in a mixture of chloroform with isopropyl alcohol (3:2, 500 mL) and washed with a solution of sodium bicarbonate (10%, two times) and with water until neutral, and the solvents were evaporated on a rotary evaporator. The resulting free base was dissolved in water (100 mL) and treated with HCl (1 N, 12.65 mL) under cooling for 15 min. Solvents and HCl were evaporated, and the oily residue was re-evaporated several times with ether until a solid was formed. Filtration afforded 5.5 g (85.0%) of amorphous title compound. Characteristics are shown in Table 1.

Bioavailability Determination. *General.* Essential amino acids for diet supplementation and choline bitartrate were purchased from Sigma. Rats (Sprague-Dawley, 40-45 g) were supplied by Charles River Inc. Montreal, PQ. Casein was obtained from Animal Nutrition Research Council Reference Protein, ICN, St. Laurent, PQ. Wheat gluten and cellulose (Alphacel) were from Teclad Diets, Madison, WI, and cornstarch was from Canada Starch Co., Toronto, ON.

Control Diets. The composition of the lysine-deficient basal diet (formulated to be adequate in all other nutrients) is shown in Table 2. The lysine-deficient basal diet contained 17.5% protein (N × 6.25) from wheat gluten and supplemented essential amino acids (arginine, histidine, methionine, threonine, and tryptophan). In nutritional studies, a nitrogen-to-protein conversion factor of 6.25 is commonly used when a diet contains more than one source of protein, such as wheat gluten plus several essential amino acids in the case of our basal diet. It has been previously demonstrated in our laboratory that the basal diet was adequate in all nutrients for rat growth method except lysine (Sarwar et al., 1988).

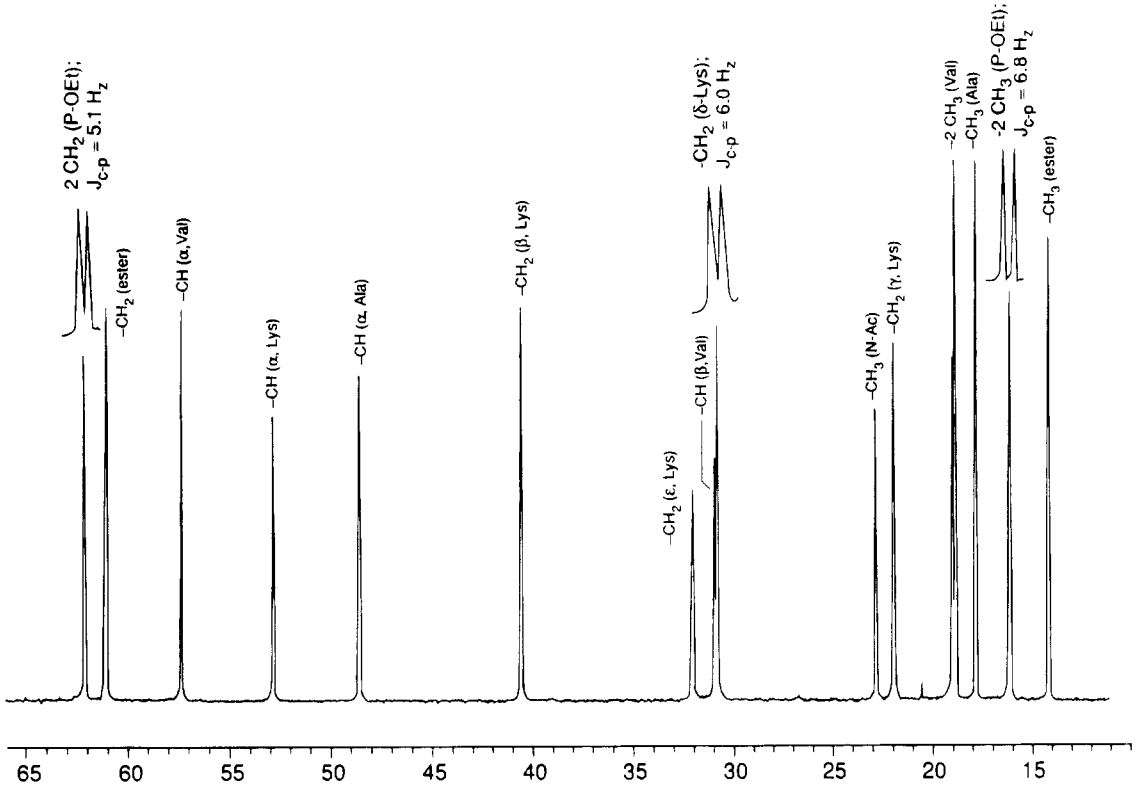

Figure 2. ^{13}C-NMR spectrum of Ac-Ala-Lys(PO_3Et_2)-Val-OEt.

Test Diets. In the present study the basal diet was supplemented either with 0.1% crystalline lysine or with each tripeptide providing 0.1% supplemental lysine. The basal and the three test diets were made isonitrogenous by the addition of a mixture of alanine, serine, and glutamic acid. Two control diets (basal plus 0.4% crystalline lysine; casein) were also included in the feeding trial. The basal plus 0.4% lysine diet was shown to meet all of the nutrient requirements for rat growth (Sarwar et al., 1988). The casein control diet contained the following (g/kg diet): casein, 200; L-methionine, 2; corn oil, 100; AIN-76 mineral mixture, 35; AIN-76-A vitamin mixture, 10; choline bitartrate, 2; cellulose, 50; sucrose, 200; cornstarch, 401. Therefore, the rat study included the feeding of seven diets: basal, basal plus 0.1% crystalline lysine, basal plus peptides providing 0.1% supplemental lysine (three diets), and two control diets.

Feeding Trial. Male weanling rats were randomly allotted to the seven experimental diets following a 2-day adaptation period. During this adaptation period, the casein control diet was fed.

The rats (eight per diet) were housed individually in stainless steel screen-bottom cages. The housing facility was maintained at 21 ± 1 °C and 50–60% relative humidity. Food and water mixture were provided ad libitum. Food cups were examined and shaken daily to ensure that there was some food always present. Paper was placed under cages to catch spilled food. Food intakes and weight gains were recorded after 1 week of test. After the test, the animals were recycled for use in another experiment.

Calculation of Bioavailability. Lysine bioavailability was calculated by comparing growth and feed efficiency (weight gain, g/100 g of food consumed) of rats fed the tripeptide diets with those fed basal plus 0.1% crystalline lysine (assuming 100% bioavailability of this source of lysine) using the following formula: (weight gain of rats fed tripeptide diet − weight gain of rats fed basal diet)/(weight gain of rats fed crystalline lysine diet − weight gain of rats fed basal diet) × 100. Data for rat growth (weight gain/100 g of food) were analyzed by one-way ANOVA and Tukey's HSD test using statistical system for personal computers (SAS, 1985).

RESULTS AND DISCUSSION

Active esters of carboxylic acids are widely used in organic chemistry for acylation of hydroxy and amino groups. Active esters of dialkylphosphoric acid, essentially representing the structure of organophosphate pesticides, react in a similar way: they phosphorylate both nucleophilic functionalities, converting hydroxy groups into phosphate esters and amino groups into phosphoramides (Eto, 1974b). According to Schrader's rule, the reaction rate between an organophosphate pesticide and a nucleophile is dependent on the electron-withdrawing potency of the leaving group and the nucleophilic strength of the substituting group (Eto, 1974b) (Figure 1). The highly nucleophilic amino group in the side chain of lysine can be expected to react with organophosphate insecticides at a high rate. It was of interest to us to determine whether or not the bioavailability of a protein-bound lysine is affected by phosphorylation by organophosphorus compounds and to what extent. To obtain precise information, we decided to carry out this work with pure synthetic peptides resembling the phosphorylated protein sequences. We chose two basic configurations, Ala-Lys-Val and Tyr-Lys-Val, using acetyl (Ac) and ethyl ester (OEt) as protecting groups of the amino and carboxyl functionalities, respectively (Table 1). We synthesized without difficulties and in high yield 22 g of pure Ac-Ala-Lys-Val-OEt (**1**) using Boc-mode synthesis in solution and EDC-mediated coupling steps. Eleven grams of this product was phosphorylated with diethyl phosphochloride using a modified process described previously for the phosphorylation of serine derivatives with diphenyl phosphochloride (Paquet, 1990). Since the nature of the phosphorus-protecting alkyl groups does not have a pronounced effect on the reactivity of organophosphate pesticides (Eto, 1974c), we have chosen the ethyl phosphate protection simply because the diethyl phosphochloride is inexpensive and easily available com-

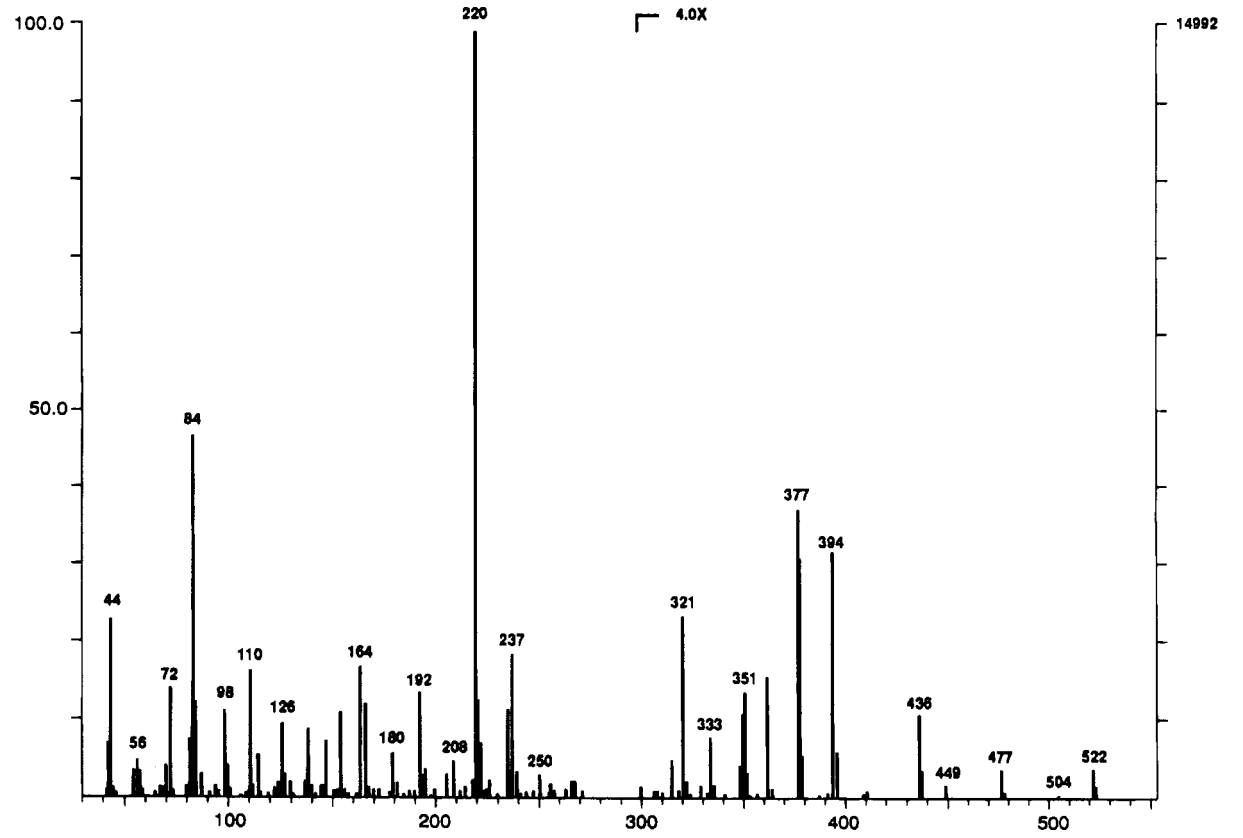

Figure 3. Mass spectrum of Ac-Ala-Lys(PO_3Et_2)-Val-OEt.

mercially. The adapted phosphorylation process proceeded with high efficiency and gave high yield (90%) of the product **2**. It was purified by crystallization from diethyl ether, giving a highly pure substance (99.7%; HPLC). Its structure was confirmed by ^{1}H-,^{13}C-, and ^{31}P-NMR and mass spectroscopy. The ^{13}C-NMR spectrum (assignment shown in Figure 2) showed the presence of carbon–phosphorus couplings on the resonances assigned to the two symmetrical $-OCH_2CH_3$ groups (62.1 ppm, CH_2, $^2J_{C-P} = 5.1$ Hz; and 16.1 ppm, CH_3, $^3J_{C-P} = 6.8$ Hz) as well as those assigned to the δ-CH_2 of the side chain of lysine (30.9 ppm, $^3J_{C-P} = 6.0$ Hz) and the ϵ-CH_2 of the side chain of lysine (32.0 ppm, J_{C-P} obscured by broadening due to the adjacent nitrogen of the terminal amino group of lysine). The ^{31}P-NMR spectrum (in CH_3OD) showed a single resonance at +11.66 ppm. One should notice the positive value of the phosphorus resonance in a phosphoramide configuration of the compound **2** (+11.66): the phosphorus resonances in phosphate esters (such as phosphoserine derivatives) are always negative (Paquet, 1990). The mass spectrum (EI mode) of **2** showed typical fragmentation with the most abundant mass unit [m/z (relative intensity) 220 (100)] resulting from the lysyl residue with the diethylphospho moiety attached to it (for details see Table 1 and Figure 3). Compounds **1** and **3** were characterized in a similar manner, and the values can be found in Table 1. Compound **3** was used in this study in its nonphosphorylated form only. The basic sequence in **3**, Tyr-Lys-Val, occurs naturally in wheat albumin (Redman, 1976), and the preparation and biological evaluation of its N-phosphorylated form will be reported later. Its characteristics are shown in Table 1. Compounds **1**–**3** were subsequently examined for bioavailability of lysine.

In the feeding experiments, growth of rats fed the basal plus 0.4% lysine diet was equivalent to that of those fed the casein control diet (Table 3), confirming a previous observation (Sarwar et al., 1988) that the basal diet used in this study was adequate in all nutrients for rat growth except lysine. Supplementation of the basal diet with 0.1% crystalline lysine or lysine from the two nonphosphorylated peptides (peptides **1** and **3**) caused substantial improvement in rat growth, while similar supplementation with the phosphorylated peptide **2** had no effect on rat growth. Lysine bioavailability values were calculated by comparing weight gains of rats fed basal plus 0.1% crystalline lysine with those of rats fed basal plus test compounds (Table 3). Values for the bioavailability of lysine in peptides **2** (phosphorylated) and **1** and **3** (nonphosphorylated) were found to be 0, 79, and 95%, respectively.

Table 3. Data on Rat Growth and Lysine Bioavailability

diet	wt gain[a] (1 week) (g/100 g of food)	Lys bioavailability (%)
basal	7.6 ± 1.5 C	
basal plus 0.1% Lys		
crystalline Lys	16.2 ± 1.8 B	100
tripeptide **1**[b]	14.4 ± 1.7 B	79
tripeptide **2**[c]	6.6 ± 1.6 C	0
tripeptide **3**[d]	15.8 ± 1.8 B	95
control		
casein	45.0 ± 2.0 A	
basal plus 0.4% Lys	46.0 ± 2.0 A	

[a] Values are means ± SEM ($n = 8$). Values in a column not sharing a common letter are significantly different ($P < 0.05$). [b] Tripeptide **1**, acetylalanyllysylvaline ethyl ester hydrochloride. [c] Tripeptide **2**, acetylalanyl N^6-diethylphospholysylvaline ethyl ester. [d] Tripeptide **3**, acetyltyrosyllysylvaline ethyl ester hydrochloride.

Phosphorylation of a lysine residue in a protein chain by organophosphate pesticides has remained entirely unexplored. In nature, this reaction does not result in any significant and measurable physiological effect; furthermore, the isolation of N-phosphorylated lysine

from the natural material is impossible due to its relatively low stability during isolation processes. Phosphorylation of lysine may contribute to nonspecific binding of organophosphate pesticides and form a part of a process of their degradation. The interaction between pesticides and cereal proteins can be expected to occur mainly during storage conditions. Association of radioactivity with proteins in stored wheat saturated with radiolabeled dichlorvos (dimethyl 2,2-dichlorovinyl phosphate, ^{14}C labeled on the methoxy moiety) was ascribed to phosphorylation of proteins (Rowlands, 1970). Mono- and dimethylphosphoserine derivatives were detected by TLC in the acid digests of individual protein fractions, but identification of phosphorylated lysine derivatives was not claimed. This is not surprising because acid digestion would most certainly hydrolyze any P–N bond present. Matthews (1988) determined rapid degradation of malathion [*O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl)phosphorodithioate, ^{14}C labeled on the succinic acid moiety)] and chlorpyrifos-methyl [*O,O*-dimethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate, ^{14}C labeled on the pyridyl moiety] in wheat under storage conditions. After 5 months, the only significant metabolic process that occurred was formation of unextractable residues (42 and 26% of the applied doses of malathion and chlorpyrifos-methyl, respectively), with their highest concentration being deposited in the germ layer. The germ layer is the physiologically most active region of wheat and contains proteins of the highest nutritional quality (Wrighley and Bietz, 1988; Hoseney, 1986). Bound residues are often formed under conditions favoring pesticide degradation (Lichtenstein et al., 1977). Thus, covalent binding, such as phosphorylation of proteins in the germ layer, would affect the biologically most important proteins.

CONCLUSION

The present study indicates that lysine was completely unavailable to weanling rats from its N^6-diethylphospho derivative bound in a synthetic model peptide, Ac-Ala-Lys(PO_3Et_2)-Val-OEt. This result would suggest an adverse effect of phosphorylation by organophosphate pesticides on the nutritive value of cereal grain proteins. Thus, work with pure synthetic compounds in this field offers information that would be unavailable during work with whole, naturally occurring polymers or with whole grains.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; Ac, acetyl; AcOH, acetic acid; DCM, dichloromethane; EDC, *N*-ethyl-*N*-(3-aminopropyl)carbodiimide hydrochloride; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Val-OEt, valine ethyl ester; Tyr(Bzl), tyrosine benzyl ether; Pd/C, palladium catalyst on charcoal (10%). Amino acid symbols represent L forms and are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.* **1972**, *247*, 977–983].

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