Nutritional and Toxicological Assessment of an N^6-Diethylphospholysyl Peptide: A Synthetic Compound Resembling the Covalently Bound Metabolite of an Organophosphate Pesticide to Protein-Bound Lysine

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A 1- and 5-week mouse growth study was conducted to evaluate the potential toxicological implications and nutritional value of a phosphorylated peptide, Ac-Ala-Lys(PO_3Et_2)-Val-OEt (a synthetic model resembling the covalently attached metabolite of organophosphate pesticide to protein-bound lysine), and the nonphosphorylated peptide Ac-Ala-Lys-Val-OEt. A basal diet, adequate in all nutrients except lysine (0.26%), was supplemented with 1 and 2% of the phosphorylated or the nonphosphorylated peptide, and with the amounts of crystalline lysine equivalent to those provided by the peptides. Relative lysine bioavailability was calculated by comparing growth of mice fed a peptide diet with that of mice fed basal plus an equivalent amount of crystalline lysine. Values for relative bioavailability of lysine (L-lysine = 100) in the phosphorylated peptide after 1 or 5 weeks of test were zero. Values for relative bioavailability of lysine in the nonphosphorylated peptide were, however, 100 and 86−99% after 1 and 5 weeks of test, respectively. The addition of the peptides had no significant adverse effects on relative mouse organ weights or most blood hematology parameters.

Keywords: *Phosphorylation; protein-bound lysine; N^6-diethylphospholysine; organophosphate pesticides; model peptides; mouse growth, organ weights; blood hematology; lysine bioavailability*

INTRODUCTION

Phosphorylation of the enzyme acetylcholinesterase by organophosphate pesticides is very well established. This phosphorylation consists of covalent attachment of dialkylphospho moiety to the serine residue in the esteratic site of the enzyme. This reaction corresponds to the physiological process of acetylation of the same serine residue by acetylcholine (Eto, 1974; Matsumura, 1975). Nonspecific binding of organophosphate pesticides is also well known to occur in mammalian tissues, probably as a metabolic disposal process (Environmental Health Criteria 63, 1986). The mechanism of this nonspecific binding has remained, however, unexplained. It is presumed that it occurs via phosphorylation of serine residues of various tissue enzymes. Metabolites of organophosphate pesticides bound to these nonvital sites are prevented from attacking the vital enzymes, such as acetylcholinesterase.

Little attention has been paid to the possibility that other protein bound reactive amino acids could also participate in the process of nonspecific binding. For example, phosphorylation of the N^6-amino group of lysine, which is a stronger nucleophile than the hydroxy group of serine, has not been considered. An organophosphate pesticide, possessing essentially the structure of an active ester (Paquet et al., 1994), could react with the N^6-terminal amino group of lysine at a rate higher than that with the hydroxy group of serine at a nonspecific site. Matthews (1988) showed that major metabolic process of ^{14}C-labeled melathion and chlorpyrifos-methyl in stored wheat is formation of unextractable residues in the germ layer. The germ layer is physiologically the most active region in wheat (Hoseney, 1986; Wrighley and Bietz, 1988). Pesticides could be thus degraded in the germ layer. Lichtenstein et al. (1977) pointed out that bound pesticide residues are often formed under conditions favoring their own degradation. Phosphorylation of various lysine-containing peptides by parathion and diazinone was studied. In all cases, the terminal amino group of lysine was phosphorylated (30−35%; Paquet, unpublished results). Thus, there is a good reason to presume that the N^6-amino group of protein-bound lysine in the natural environment could be potentially phosphorylated by organophosphate pesticides.

A model peptide containing N^6-diethylphospholysine on an internal position, Ac-Ala-Lys(PO_3Et_2)-Val-OEt, and two nonphosphorylated counterparts, Ac-Ala-Lys-Val-OEt and Ac-Tyr-Lys-Val-OEt, were synthesized in high yields and fully characterized by physiochemical constants, HPLC, NMR, and mass spectra (Paquet et al., 1994). A preliminary feeding study suggested 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, whereas values for bioavailability of lysine in the two nonphosphorylated peptides, Ac-Ala-Lys-Val-OEt and Ac-Tyr-Lys-Val-OEt, were 79 and 95%, respectively (Paquet et al., 1994).

The feeding study of Paquet et al. (1994) was of short duration (1 week), and the phosphorylated model peptide was tested at a low dietary level (0.3%). Further studies including longer duration and higher dietary

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S0021-8561(95)00599-1 CCC: $12.00 Published 1996 by the American Chemical Society

Table 1. Composition of Basal Diet (As Is Basis)[a]

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

[a] The same basal diet was used in the study of Paquet et al. (1994). [b] Wheat gluten contained 87.69% crude protein (N × 6.25). [c] American Institute of Nutrition (1977). [d] American Institute of Nutrition (1980). [e] Indispensable 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); and Arg (76.9).

test levels are required to assess the safety and nutritional quality of the phosphorylated model peptide. Therefore, a 5-week mouse growth experiment was conducted in the present investigation to assess the possible adverse effects of feeding 1 and 2% of the model phosphorylated peptide, Ac-Ala-Lys(PO_3Et_2)Val-OEt. The nonphosphorylated counterpart, Ac-Ala-Lys-Val-OEt, was also included for comparison.

MATERIALS AND METHODS

Preparation of Peptides. The two peptides, Ac-Ala-Lys-(PO_3ET_2)-Val-OEt and Ac-Ala-Lys-Val-OEt, were prepared as described previously (Paquet et al., 1994).

Mouse Growth Experiment. *General.* Male weanling mice (10−11 g) were supplied by Charles River Inc., Montreal, PQ, Canada. Casein (ANRC, Animal Nutrition Research Council Reference Protein), wheat gluten, cellulose (alphacel), cornstarch, crystalline amino acids, and choline bitartarate were purchased from commercial sources as noted previously (Paquet et al., 1994).

Diets. The composition of the lysine-deficient basal diet is shown in Table 1. The basal diet has been shown to be adequate in all nutrients for mouse growth except lysine (Ujiie et al., 1993). The lysine-deficient basal diet contained 17.5% protein (N × 6.25) from wheat gluten and supplemented indispensable amino acids (Arg, His, Met, Thr, and Trp). The basal diet was supplemented with two levels (1 and 2%) of the phosphorylated tripeptide, Ac-Ala-Lys(PO_3Et_2)-Val-OEt, the nonphosphorylated tripeptide, Ac-Ala-Lys-Val-OEt·HCl (test diets). The 1 and 2% addition of the phosphorylated tripeptide provided 0.28 and 0.56% supplemental lysine, respectively. Similarly, the 1 and 2% addition of the nonphosphorylated tripeptide provided 0.35 and 0.70% supplemental lysine, respectively. Therefore, the basal diet was supplemented with 0.28, 0.35, 0.56, and 0.70% crystalline lysine to serve as standard diets. A casein control was also included in the feeding study. The casein control diet contained the following (g/kg of diet): ANRC casein (200); L-methionine (2); corn oil (100); AIN-76 mineral mixture (35); AIN-76A vitamin mixture (10); choline bitartarate (2); cellulose (50); sucrose (200); and cornstarch (401). Therefore, the mouse growth study included the feeding of 10 diets (basal, four standard, four test, and a casein control). All the test diets were made isonitrogenous by the addition of a mixture of alanine, serine, and glutamic acid.

Feeding Trial. Male weanling mice were randomly allotted to the 10 experimental diets following a 2-day adaptation period during which the casein control diet was fed. The mice (10/diet) were housed individually in stainless steel, screen-bottomed cages in a temperature- and humidity-controlled housing facility (Ujiie et al., 1993). The feeding trial lasted for 5 weeks. Records of weekly body weight and food consumption were kept. After the feeding trial, mice were anesthetized with 3% halothane in oxygen and then exsanguinated for the collection of blood, liver, kidneys, and spleen samples.

Calculation of Bioavailability. Relative lysine bioavailability after 1 and 5 weeks of the feeding trial was calculated by the following formula: (weight gain of mice fed tripeptide diet − weight gain of mice fed basal diet)/(weight gain of rats fed crystalline lysine diet − weight gain of mice fed basal diet) × 100.

Blood Hematology Analysis. For the hematology component of the study, blood samples were collected individually from five mice per diet group. The following peripheral blood hematology parameters were analyzed with a Coulter Counter Model S-Plus IV (Coulter Electronics Inc., Hialeah, FL): red blood cell count (RBC); hemoglobin concentration (Hb); hematocrit (Hct); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); red cell distribution width (RDW); platelet count; mean platelet volume (MPV); platelet distribution width (PDW); leukocyte concentration (WBC); and absolute leukocyte differential counts: eosinophils, neutrophils segmented, neutrophil bands, lymphocytes, and monocytes. The differential leukocyte concentrations were obtained by multiplying the percentage of leukocyte cell type in 200 cells by the total leukocyte concentration (WBC). The parameter neutrophil bands was not analyzed because most of the values were zero.

Statistics. Data for rat growth (weight gain/25 or 100 g of food), organ weights (liver, kidneys, and spleen), and blood hematology parameters were analyzed by one-way ANOVA and Tukey's Studentized Range Test with a statistical system for personal computers (SAS, 1985).

RESULTS AND DISCUSSION

The data on mouse growth and lysine bioavailability are shown in Table 2. The basal diet was formulated to contain insufficient (0.26%) lysine but adequate amounts of all other nutrients. Therefore, the addition of crystalline lysine (which is assumed to be 100% bioavailable) was expected to promote growth. The growth (1 or 5 weeks) of mice fed basal plus crystalline lysine diets was equivalent to that of those fed the casein control diet, confirming a previous observation (Ujiie et al., 1993) that the basal diet used in this investigation was adequate in all nutrients for mouse growth except lysine. The basal diet used in the present study (Table 1) was similar to that used by Ujiie et al. (1993); the only nutritionally minor differences being more starch (at the expense of sucrose) and 0.2% supplemental L-glutamic acid (added for making diets isonitrogenous) in the latter case. In the present study, the test diets were made isonitrogenous by the addition of a mixture of dispensable amino acids (L-alanine, L-serine, and L-glutamic acid).

The growth rates (1 or 5 weeks) of mice fed the basal plus phosphorylated peptide (1 or 2%) diets were significantly lower than of those fed the basal plus crystalline lysine or nonphosphorylated peptide (1 or 2%) diets. In addition, the 1- and 5-week growth rates of mice fed basal plus 2% phosphorylated peptide were ∼45 and 11% lower compared with those fed the basal diet, suggesting a growth-inhibiting effect of the phosphorylated peptide, especially during the early growth period of mice when the requirements for amino acids are more intense. Protein and amino acid requirements of rodents [rats and mice are known to decline rapidly with age after weaning (NRC, 1995)]. For example, the protein requirement of rats declined from 28% of the diet at 30 days of age (weaning) to 10% at 50 days of age (20 days after weaning). A similar decline in protein and amino acid requirements with age could be expected for mice.

Relative lysine bioavailability (a measure of absorption and utilization for protein synthesis) values (Table 2) were calculated by comparing weight gains of mice fed basal plus crystalline lysine with those of mice fed basal plus test peptides providing the same amount of

Table 2. Data on Mouse Growth and Relative Lysine Bioavailability

diet	wt gain[a] 1 week (g/25 g of food)	wt gain[a] 5 weeks (g/100 g of food)	rel Lys bioavailablity (%)[b] 1 week	rel Lys bioavailablity (%)[b] 5 week
basal	4.07 ± 0.46B	11.38 ± 1.30A	–	–
basal + 0.28% Lys				
crystalline Lys	7.05 ± 0.63C	13.90 ± 1.26B	100	100
phosphorylated peptide (1%)	3.93 ± 0.50B	11.27 ± 1.20A	0	0
basal + 0.35% Lys				
crystalline Lys	6.91 ± 0.62C	13.26 ± 1.18B	100	100
nonphosphorylated peptide (1%)	6.95 ± 0.61C	13.24 ± 1.24B	100	99
basal + 0.56% Lys				
crystalline Lys	7.86 ± 0.60C	13.96 ± 1.18B	100	100
phosphorylated peptide (2%)	2.22 ± 0.31A	10.07 ± 1.25A	0	0
basal + 0.7% Lys				
crystalline Lys	7.93 ± 0.66C	13.79 ± 1.30B	100	100
nonphosphorylated peptide (2%)	7.95 ± 0.72C	13.46 ± 1.24B	100	86
casein control	7.88 ± 0.58C	13.88 ± 1.20B	–	–

[a] Values are means ± SD ($n = 10$); values in a column not sharing a common letter are significantly ($p < 0.05$) different. [b] Values below zero were considered zero, and values >100 were considered 100.

Table 3. Data on Relative Mouse Organ Weights (5-Week Study)

diet	liver[a]	kidneys[a]	spleen[a]
basal	5.10 ± 0.48	1.79 ± 0.27	0.30 ± 0.08
basal + 0.28% Lys			
crystalline Lys	5.36 ± 0.73	1.79 ± 0.17	0.25 ± 0.05
phosphorylated peptide (1%)	5.10 ± 0.44	1.75 ± 0.16	0.27 ± 0.05
basal + 0.35% Lys			
crystalline Lys	5.39 ± 0.47	1.72 ± 0.28	0.28 ± 0.08
nonphosphorylated peptide (1%)	5.57 ± 0.56	1.74 ± 0.14	0.27 ± 0.04
basal + 0.56% Lys			
crystalline Lys	5.64 ± 0.64	1.61 ± 0.17	0.23 ± 0.03
phosphorylated peptide (2%)	5.36 ± 0.53	1.78 ± 0.17	0.28 ± 0.03
basal + 0.70% Lys			
crystalline Lys	5.36 ± 0.22	1.68 ± 0.14	0.25 ± 0.04
nonphosphorylated peptide (2%)	5.28± 0.55	1.68 ± 0.19	0.21 ± 0.08
casein control	5.17 ± 0.62	1.65 ± 0.13	0.28 ± 0.10

[a] Values are means ± SD ($n = 10$) and are expressed as g/100 g body weight.

Table 4. Data on Selected Mouse Blood Hematology Parameters[a,b]

diet	RBC ($\times 10^{12}$/L)	Hb (g/L)	Hct	MCV (fL)	RDW (%)
basal	8.67 ± 0.16C	136.60 ± 3.56D	0.43 ± 0.01C	49.66 ± 1.76AB	13.90 ± 0.66A
basal + 0.28% Lys					
crystalline Lys	9.69 ± 0.73AB	150.20 ± 7.15AB	0.47 ± 0.02ABC	48.04 ± 1.51AB	12.68 ± 0.67ABC
phosphorylated peptide (1%)	8.83 ± 0.38BC	137.00 ± 4.69CD	0.43 ± 0.01C	48.76 ± 1.22AB	13.13 ± 0.28ABC
basal + 0.35% Lys					
crystalline Lys	9.58 ± 0.44ABC	149.40 ± 6.94ABC	0.47 ± 0.01AB	49.22 ± 1.16AB	12.92 ± 0.49ABC
nonphosphorylated peptide (1%)	9.31 ± 0.47ABC	147.60 ± 9.28ABCD	0.46 ± 0.02ABC	49.40 ± 0.82AB	12.42 ± 0.55C
basal + 0.56% Lys					
crystalline Lys	9.81 ± 0.38A	148.60 ± 3.20ABCD	0.47 ± 0.00ABC	47.46 ± 1.53B	12.74 ± 0.18ABC
phosphorylated peptide (2%)	9.13 ± 0.50ABC	142.60 ± 7.19BCD	0.45 ± 0.02BC	48.96 ± 1.32AB	13.75 ± 0.91AB
basal + 0.70% Lys					
crystalline Lys	9.56 ± 0.46ABC	150.20 ± 4.43AB	0.47 ± 0.02ABC	49.10 ± 1.08AB	12.46 ± 0.56C
nonphosphorylated peptide (2%)	9.09 ± 0.31ABC	144.00 ± 2.16BCD	0.46 ± 0.01ABC	50.45 ± 0.12A	12.70 ± 0.47ABC
casein control	9.85 ± 0.44A	156.60 ± 4.72A	0.49 ± 0.02A	49.96 ± 0.42AB	12.52 ± 0.67BC

[a] RBC, red blood cell count; Hb, hemoglobin concentration; Hct, hematocrit; MCV, mean corpuscular volume; RDW, red cell distribution width. [b] Values are means ± SD ($n = 5$); values in a column not sharing a common letter are significantly ($p < 0.05$) different.

lysine. Actual values for the bioavailability of lysine in the phosphorylated peptide tested at 1% at 1 and 5 weeks were −5 and −4%, respectively. In contrast, the actual values for bioavailability of lysine in the phosphorylated peptide tested at 2% at 1 and 5 weeks were −49 and −51%, respectively. In nutritional studies, it is customary to consider relative bioavailability values (L-lysine = 100) of below zero as zero, and of above 100 as 100. The negative bioavailability values would, however, suggest an antinutritional effect of the phosphorylated peptide on protein digestion and/or amino acid utilization of the basal diet, especially at the 2% addition. Lysine was the growth-limiting nutrient in the basal diet, so the reduced growth caused by the addition of the phosphorylated peptide would suggest an adverse effect on the absorption and/or utilization of lysine (for protein synthesis) present in the basal diet. Actual values for the relative bioavailability of lysine in the nonphosphorylated peptide were, however, 100−101 and 86−99% after 1 and 5 weeks of test, respectively (Table 2).

The data on relative organ (liver, kidneys, and spleen) weights after 5 weeks of test are shown in Table 3. The treatments had no significant effects on the relative organ weights (livers, 5.10−5.64 g/100 g body weight; kidneys, 1.65−1.79 g/100 g of body weight; spleens, 0.21−0.28 g/100 g of body weight; Table 3). Several authors (Shirley, 1977; Weil and Gad, 1980) have reported a parallel relationship between increases in organ weight and increases in body weights, except for

cases of obesity and starvation, as well as changes associated with aging in chronic bioassays. The present study involved young, growing mice and neither obesity nor starvation was a factor regarding the experimental treatments. Therefore, we chose to compare relative organ weights (g/100 g of body wt) for treatments because we were aware of the differences in growth rates (Table 2). Three organs (liver, kidneys, and spleen) were studied for particular reasons. The liver was studied because it is the first organ that encounters all absorbed material from the gastrointestinal tract, and has been shown to respond in a number of ways to a toxicological insult. One crude method of ascertaining whether a possible toxicological response occurred in the liver is to evaluate its relative weight. If there had been an increase in relative liver weight, then further evaluation via histological examination would have been warranted. The kidney was studied because it is an excretory organ in which a toxicological metabolite arising from the test treatment would probably be found at its greatest concentration. Finally, the spleen was studied because it is an organ that provides a very crude insight into whether the test treatment may have affected the immune system.

The dietary treatments had no significant effects on many blood hematology parameters including WBC ($3.35 \pm 2.18-5.86 \pm 1.58$ x10^9/L), MCH ($15.36 \pm 0.50-15.90 \pm 0.31$ pg), MCHC ($316.80 \pm 6.13-320.80 \pm 8.35$ g/L), platelets ($1137.75 \pm 75.09-1405.20 \pm 243.24 \times 10^9$/L), MPV ($4.28 \pm 0.13-5.10 \pm 0.29$ fL), PDW ($15.75 \pm 0.46-17.33 \pm 0.90$%), eosinophils ($0.05 \pm 0.03-0.15 \pm 0.14 \times 10^9$/L), neutrophils segmented, lymphocytes ($2.13 \pm 0.78-4.39 \pm 0.97 \times 10^9$/L), and monocytes ($0.10 \pm 0.09-0.25 \pm 0.09$).

The dietary treatments did have significant effects on five blood hematology parameters (Table 4). The RBC, Hb, and Hct concentrations were larger in the crystalline lysine diet groups than in the phosphorylated peptide or nonphosphorylated peptide groups, although the difference between diet groups at the same lysine level was statistically significant only for Hb concentrations at 0.28% level. The RBC, Hb, and Hct concentrations were significantly greater in the casein group compared with the basal diet group (Table 4). For MCV and RDW, there were differences among pairs of diet groups but not between diet groups at the same lysine level. For RDW, the basal diet group mean was significantly larger than the mean of the casein diet.

SUMMARY AND CONCLUSION

The present mouse growth data confirm the results of an earlier preliminary rat study (Paquet et al., 1994) about complete unavailability of lysine from its N^6-diethylphospho derivative bound in the synthetic model peptide Ac-Ala-Lys(PO_3Et_2)-Val-OEt. This observation would suggest an adverse effect of potential phosphorylation by organophosphate pesticides on the nutritional quality of proteins in cereal crops. The addition of 1 or 2% of the phosphorylated peptide exerted no adverse effects on relative mouse organ (liver, kidneys, and spleen) weights and most blood hematology parameters, but the 2% addition of the phosphorylated peptide did suppress body weight gains during the early growth period of mice (first week of study), suggesting an adverse effect of the phosphorylated peptide on protein digestion and/or amino acid utilization. The addition of 1 and 2% of the phosphorylated peptide would correspond to 50 and 100%, respectively, of the lysine in cereal grains containing 0.56% lysine.

ACKNOWLEDGMENT

The technical assistance of Mrs. D. Miller and Mrs. S. Fernie in the preparation of the manuscript is appreciated.

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Received for review September 5, 1995. Accepted May 16, 1996.[⊗]

JF9505990

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1996.