we observed at the conclusion of the calorimeter runs. However, protein aggregation following denaturation is normally of such small magnitude in relation to the endotherm produced by denaturation that it is generally ignored (Donovan and Ross, 1973).

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Poly-L-lysine and Multioligo(L-methionyl)poly-L-lysine as Nutritional Sources of Essential Amino Acids

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Poly-L-lysine and the corresponding branched-chain polypeptide multioligo(L-methionyl)poly-L-lysine were synthesized by the *N*-carboxy anhydride method, and their nutritional properties were investigated by in vitro and in vivo methods. The extent of modification of poly-L-lysine, consistent with good availability of both amino acids, could be estimated by an in vitro enzyme digestion procedure. Lysine and methionine were found to be rapidly released from the polymer provided that their molar ratio was not beyond 2. Their bioavailability was further confirmed by rat feeding experiments using a 10% basal protein diet supplemented with either free methionine and free lysine or multioligo(L-methionyl)-poly-L-lysine. In both cases, the methionine to lysine molar ratios were 0.85 and 1.30, respectively. The synthetic branched-chain polymer was found to be as effective as the corresponding free amino acids in meeting the rat requirements for growth.

The nutritional value of lower quality food proteins may be improved through fortification with essential amino acids supplied either as the free form of as covalently attached to proteins. As a matter of fact, chemical and enzymatic methods have been successfully applied to food proteins to enhance their content in one or more limiting amino acids over the past few years (Puigserver et al., 1982; Yamashita et al., 1979). More recently, polymerization of L-methionine onto the ϵ-amino groups of casein and β-lactoglobulin by the *N*-carboxy anhydride method resulted in the preparation of modified proteins in which as much methionine as 30% of protein weight was covalently attached in the form of polymethionine of an average chain length of eight residues (Gaertner and Puigserver, 1984a). The bioavailability of covalently attached methionine could be derived from in vitro enzyme digestion experiments in which the successive hydrolysis of methionine polymers by gastric, pancreatic, and intestinal enzymes was quantitatively estimated (Gaertner and Puigserver, 1986). An alternative means to prevent the possible deteriorative reactions occurring in food proteins when they are sup-

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plemented with free essential amino acids may be the use of synthetic amino acid polymers provided that they are efficiently hydrolyzed by the enzymes of the digestive tract.

Synthetic branched-chain polyamino acids have been extensively used as antigens to study the molecular basis of their immunological reactions as well as macromolecular carriers of various biologically active materials (Sela, 1966; Muller et al., 1982; Audibert et al., 1982; Arnold, 1985). By contrast, the nutritive value of poly-L-lysine, exclusively, has been investigated with lysine-deficient protein diets in the rat (Newman et al., 1980). However, the fact that this polymer was effective in meeting lysine requirement could easily be predicted since poly-L-lysine had already been shown to be readily hydrolyzed by trypsin into dilysine, almost exclusively (Waley and Watson, 1953).

Since methionine and lysine are usually the first limiting amino acids in proteins from plants, single cells, and other less conventional sources, the aim of this work has been to polymerize L-lysine and then L-methionine onto poly-L-lysine and, finally, to investigate the biological availability of the resulting multioligo(L-methionyl)poly-L-lysine.

MATERIALS AND METHODS

Materials. L-Methionine, L-lysine, N^{ϵ}-carbobenzoxy-L-lysine were from Sigma Chemical Co., St Louis, MO, while phosgene was from l'Air Liquide, Lyon, France. All anhydrous solvents (puran grade) used in polymer synthesis were from SDS (Solvants, Documentation, Synthèse), Peypin, France. Hog pepsin was supplied by Worthington Biochemicals Corp. Freehold, NJ, whereas calf pancreatic juice and pure hog aminopeptidase N were gifts from P. Thivend, Theix, and S. Maroux, Marseille, France, respectively. Basal diet containing 10% protein was obtained from UAR (Usine d'Alimentation Rationnelle Villemoisson-sur-Orge, France). Male weanling Wistar rats weighing between 50 and 60 g were purchased from Iffa-Credo, l'Arbresle, France.

Methods. *Synthesis of Poly-L-lysine.* N^{ϵ}-(Benzyloxycarbonyl)-N^{α}-carboxy-L-lysine anhydride was synthesized by reacting the N^{ϵ}-protected amino acid suspended in anhydrous tetrahydrofuran with phosgene (Fasman et al., 1961; Hirschmann et al., 1971) in a hood with a good draft as previously described (Gaertner and Puigserver, 1984a) and further purified by repeated crystallization in ethyl acetate–hexane. Subsequent polymerization of the *N*-carboxy-α-amino acid anhydride was performed in anhydrous dioxane with sodium methylate as initiator (1:100 weight ratio of initiator to *N*-carboxy anhydride). The reaction proceeded at room temperature, and after a 3-day period the viscous solution was directly freeze-dried. Removal of benzyloxycarbonyl groups from the resulting polymer was achieved by treatment with 33% hydrogen bromide in acetic acid (Ben Ishai and Berger, 1959). Precipitated poly-L-lysine hydrobromide was washed with ethyl ether and dried in vacuo. It was then dissolved in water, extensively dialyzed against distilled water, and finally freeze-dried.

Synthesis of Multioligo(L-methionyl)poly-L-lysine. Polymerization of L-methionine onto the ϵ-amino groups of poly-L-lysine was carried out in aqueous buffered solutions by the *N*-carboxy anhydride method as described for proteins (Gaertner and Puigserver, 1984a). Branched-chain polymers containing polymethionine of an average chain length of up to five residues were separated from reaction byproducts by dialysis and finally freeze-dried.

Viscosity Determination. A Cannon Ubbelohde (semimicro size 50) viscometer was used to measure the intrinsic viscosity $[\eta]$ of poly-N^{ϵ}-(benzyloxycarbonyl)-L-lysine in dimethylformamide solution. The degree of lysine polymerization was calculated as $10^7[\eta]/2.24^{0.8} \times {}^1/_{262}$ according to Daniel and Katchalski (1962).

Amino Acid Analysis and Amino Group Determination. Branched-chain polymers were characterized by their amino acid composition and free ϵ-amino group content. The amino acid composition was determined with a Beckman Model 120 C autoanalyzer equipped with an ICAP 10 computer, following hydrolysis of the polymer with distilled 5.6 N HCl at 110 °C for 24 h. Methionine was determined as methionine sulfone after performic acid oxidation of the polypeptide (Hirs, 1956; Moore, 1963). Chemical modification of the free lysyl residues with sodium nitrite into α-amino-ϵ-caproic acid (Anfinsen et al., 1962) was used to assess complete removal of the protecting group following hydrobromic acid treatment and to estimate the extent of lysine modification after polymerization of L-methionine on poly-L-lysine. The lithium citrate buffers for physiological fluids analysis were used for blood and muscle samples (Stein and Moore, 1954).

Table I. Composition of the Basal Diet Used in Rat Feeding Studies and Final Content in Essential Amino Acids

compn	% dry matter	essential amino acid	% dry matter[a]
gluten[b]	10	threonine	0.55
gelatin	5	valine	0.34
cornstarch	50	methionine[c]	0.25
sucrose	25	isoleucine	0.28
corn oil	5	leucine	0.38
mineral mix	4	phenylalanine	0.30
vitamin mix	0.5	lysine	0.28
threonine	0.3	histidine	0.15
tryptophan	0.2	tryptophan	0.25

[a] Analytical results following acid hydrolysis. [b] Providing 5% crude protein equivalent (N × 6.25). [c] Including cysteine (0.07%).

In Vitro Digestibility Studies. Polylysine samples containing a number of covalently attached oligomethionine of different average chain lengths were successively digested by hog pepsin, activated pancreatic juice, and hog aminopeptidase as already detailed by Gaertner and Puigserver (1986). The resulting hydrolysates were 3-fold diluted with a 0.2 M sodium citrate buffer, and their amino acid composition was subsequently determined with the autoanalyzer.

Nutritional Studies with Rats. Weanling Wistar male rats were housed in wire-bottom cages and had free access to food and water. They were fed a stock diet for 5 days and then separated into groups of 7 or 10 animals each (depending on the experiment) of approximately equal mean initial weight and fed the experimental diets. The cages were placed in an air-conditioned room maintained at 20 °C with a 12-h light–dark cycle. Body weight and food intake were recorded every 2 or 3 days throughout the test period. The composition of the basal diet used throughout this study is given in Table I. This lysine- and methionine-deficient protein diet was supplemented with both amino acids either in the free form or as the synthesized polymers so that the levels of lysine and methionine in the experimental diets were 0.85% and 0.59% on a weight basis, respectively. Those are known to be the usual amino acid levels required to achieve maximal growth rate in the rat. Comparisons between PER mean values were performed by a two-tailed Student's *t*-test, and $P < 0.05$ was considered significant.

Blood and Muscle Sampling. Rats were sacrificed by decapitation under ether anesthesia. Blood was immediately mixed with cold ethanol (−10 °C), and hind limbs were rapidly excised and frozen in liquid nitrogen. Determination of free amino acids in whole blood and muscles in the pooled samples from each experimental group of animal was carried out as described by Pawlack and Pion (1968).

RESULTS

Synthesis of Multioligo(L-methionyl)poly-L-lysine. Poly-L-lysine was used as a multivalent initiator in the polymerization of *N*-carboxy-L-methionine anhydride, giving rise to branched-chain poly-L-lysine in which a number of oligomethionine side chains were covalently attached to the polylysine backbone.

The poly-L-lysine moiety was obtained as an hydrobromide salt containing about 70% L-lysine, after complete removal of benzyloxycarbonyl protecting groups and subsequent dialysis against water. Its average molecular weight, as determined by viscosity studies on the still-protected polymer in dimethylformamide (Daniel and Katchalski, 1962), was estimated to be about 100K. The covalent attachment of poly-L-methionine to poly-L-lysine

Table II. Extent of Modification of Poly-L-lysine with *N*-Carboxy-L-methionine Anhydride

molar ratio of reagent to amino groups	Met/Lys content, mol/mol	modified Lys residues,[a] %	polymethionine av chain length, residues
	Polyaddition at pH 6.5[b]		
1	0.55	27	2.0
1.5	0.83	30	2.7
3	1.90	49	3.8
5	3.40	70	5.0
	Stepwise Synthesis at pH 10.2[c]		
1 × 1	0.45	45	1.0
1 × 1.2	0.53	50	1.0
3 × 1	2.10	66	3.2

[a] Determined as those residues that were not transformed into α-amino-ε-caproic acid by reaction of the polymer with sodium nitrite. [b] Single addition of the reagent in a 0.1 M sodium citrate buffer. [c] Number of successive additions × molar excess of reagent in a 0.1 M bicarbonate buffer.

Table III. Methionine and Lysine Release in the Form of Free Amino Acids from the Multichain Polymer by Pepsin, Activated Pancreatic Juice, and Aminopeptidase N

Met/Lys content of the polymer, molar ratio	amino acid released, %	
	Met	Lys
0.55[a]	45	44
1.90[a]	63	53
3.40[a]	18	5
0.45[b]	56	54
0.53[b]	54	60
2.10[b]	2	5

[a] Polymer prepared under polymerization at pH 6.5. [b] Polymer prepared by stepwise synthesis conditions at pH 10.2.

was performed in aqueous buffers at pH 6.5 or 10.2, as described for proteins (Gaertner and Puigserver, 1984a). Table II shows that 27–70% of the ε-amino groups of poly-L-lysine were acylated by polymethionine side chains containing up to five residues. Acylation of all the lysyl residues was found not to be possible by successive additions of the reagent at pH 10.2 as was the case with proteins (Gaertner and Puigserver, 1984a), mainly because of the poor solubility of polylysine at pH values higher than neutrality.

In Vitro Enzyme Digestion of Amino Acid Polymers. Table III shows the enzymatic digestion of multioligo(L-methionyl)poly-L-lysine as influenced by the extent of modification of the polylysine backbone. As indicated, the release of both methionine and lysine in the form of free amino acids was severely decreased when the amount of covalently attached methionine exceeded twice the content in lysine of the branched-chain polymer. Moreover, when the molar ratio of methionine to lysine was beyond 2, acylation of a higher number of lysyl residues under stepwise synthesis conditions at pH 10.2 was found to have a more pronounced effect on digestibility than covalent attachment of longer methionine polymers on fewer lysyl residues. This observation could actually be related to the lower solubility of the polymers synthesized at pH 10.2 as well as to a significant decrease in the accessibility of the poly-L-lysine core to proteolytic enzymes. Consequently, this type of polymer was not further investigated.

Nutritional Studies with Rats. Two distinct sets of experiments were performed with two different samples of multioligo(L-methionyl)poly-L-lysine, which were both synthesized under polymerization conditions at pH 6.5. Their molar ratios in methionine to lysine (0.85 and 1.30, respectively) were such that a significant decrease in their digestibility in vivo could be ruled out (see above). The nutritional value of each branched-chain polymer was compared with that of polylysine supplemented with free methionine as well as with a mixture of crystalline lysine and methionine. The PER (protein efficiency ratio) response of rats either fed the control diet or a given experimental diet is shown in Table IV. It should be mentioned here that methionine and lysine requirements for young growing rats were fulfilled (0.59 and 0.85%, respectively) whether the basal diet was supplemented by the free amino acids or by polymethionine covalently attached to polylysine. However, an excess of methionine with respect to requirement was quite often imposed by the chemical composition of the synthesized multioligo(L-methionyl)poly-L-lysine, in the second set of experiments: basal diet supplemented with free methionine and lysine (D'_{1b}) as well as with the branched-chain polymer (D'_3). The growth response of rats to polylysine and the corresponding branched-chain polymer is also illustrated in Figure 1.

As shown in both Table IV and Figure 1, supplementation of the basal diet with free lysine and free methionine (D_1, $D'_{1a,b}$), polylysine and free methionine (D_2, D'_2), or the branched-chain polymer (D_3, D'_3) significantly improved ($P < 0.01$) to about the same extent the growth rate of weanling rats and nutritional value of the basal protein diet. However, supplementation of the basal diet with a rather high level of free methionine (0.99%) resulted in some decrease in both food intake and PER value in the animals fed D'^{1b} diet as compared to diet D'_3, which was supplemented to the same extent with multioligo(L-

Table IV. PER Response of Weanling Rats to Poly-L-methionine Covalently Attached to Poly-L-lysine

diet description	diet no.	diet composition, % w/w		wt gain, g/day	protein intake, g/day	PER[a]
		Met + Cys	Lys			
		First Experiment[b]				
basal diet alone	D_0	0.25	0.28	2.95 ± 0.41	1.49 ± 0.19	1.98 ± 0.12
+ methionine and lysine	D_1	0.72	0.85	3.72 ± 0.92	1.47 ± 0.22	2.67 ± 0.35
+ polylysine and methionine	D_2	0.72	0.85	5.16 ± 0.62	1.81 ± 0.22	2.86 ± 0.14
+ branched-chain polymer[c]	D_3	0.72	0.85	3.97 ± 0.75	1.56 ± 0.18	2.52 ± 0.25
		Second Experiment[d]				
basal diet alone	D'_0	0.25	0.28	2.55 ± 0.32	1.60 ± 0.18	1.60 ± 0.24
+ methionine and lysine	D'_{1a}	0.59	0.85	4.44 ± 0.54	1.64 ± 0.19	2.70 ± 0.13
+ methionine and lysine	D'_{1b}	0.99	0.85	3.54 ± 0.38	1.56 ± 0.14	2.28 ± 0.24
+ polylysine and methionine	D'_2	0.59	0.85	4.59 ± 0.95	1.73 ± 0.12	2.65 ± 0.28
+ branched-chain polymer[e]	D'_3	0.99	0.85	4.37 ± 0.82	1.79 ± 0.13	2.45 ± 0.15

[a] Protein efficiency ratio calculated as the weight gain per unit weight of protein consumed. [b] Over a 14-day period with groups of seven rats each. All values are means ± SEM. [c] Multioligo(L-methionyl)poly-L-lysine with a methionine to lysine molar ratio of 0.85. [d] Over an 18-day period with groups of 10 rats each. All values are means ± SEM. [e] As in *c* with a methionine to lysine molar ratio of 1.30.

Table V. Amino Acid Pattern in Blood and Muscle from Rats Fed the Basal Protein Diet Alone or Supplemented with Free Methionine and Free Lysine or with Multioligo(methionyl)polylysine[a]

amino acid, mg/100 g	blood[b]				muscle			
	0	+ Met + Lys	+ Met + poly-Lys	+ poly-Met + poly-Lys[c]	0	+ Met + Lys	+ Met + poly-Lys	+ poly-Met + poly-Lys[c]
Asp	0.52	0.49	0.60	0.49	7.37	5.09	5.31	5.28
Thr	4.22	2.09	4.94	4.56	23.99	19.71	21.03	18.08
Ser	4.57	1.87	4.76	4.78	34.12	27.56	24.44	25.71
Glu	7.43	4.49	7.95	8.73	89.04	71.93	76.29	80.21
Gly	4.42	3.30	6.33	6.92	97.29	67.83	93.73	78.19
Ala	3.14	1.80	4.65	4.05	33.63	28.10	27.10	20.50
Lys	1.85	3.88	6.64	7.41	3.74	46.36	44.45	20.78

[a] Diets are those corresponding to the first set of experiments in Table IV. [b] Calculations based on a dry matter content of 20% (w/w) in blood. [c] Multioligo(L-methionyl)poly-L-lysine with a methionine to lysine molar ratio of 0.85.

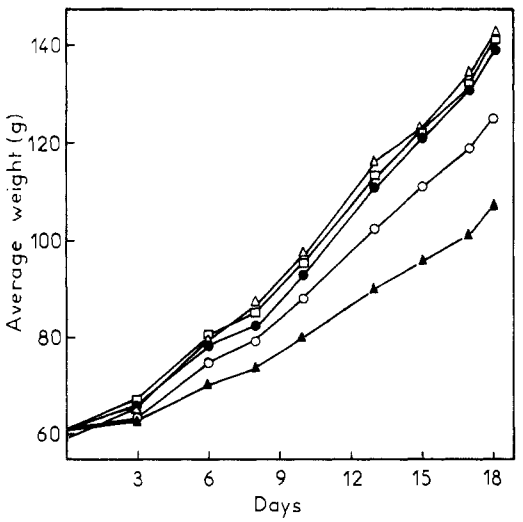

Figure 1. Growth response of rats fed a 10% basal protein diet either alone (D'_1, ▲) or supplemented with free methionine and free lysine (D'_{1a}, D'_{1b}), the methionine levels being 0.59% (□) and 0.99% (○), polylysine and free methionine (D'_2, △) or multioligo(L-methionyl)poly-L-lysine (D'_3, ●), corresponding to the second set of experiments in Table IV. Each experimental value is the mean of 10 determinations (groups of 10 rats each).

methionyl)poly-L-lysine. In both sets of experiments, supplementation of the basal diet with either polylysine and free methionine (D_2, D'_2) or the branched-chain polylysine (D_3, D'_3) gave quite comparable PER values. Thus, poly-L-lysine and multioligo(L-methionyl)poly-L-lysine may be considered as effective sources of lysine and of methionine and lysine, respectively.

Free Amino Acid Patterns in Blood and Muscle. The blood and muscular tissue levels of some free amino acids from rats fed the basal protein diet supplemented with methionine and lysine either in the free form or as multioligo(L-methionyl)poly-L-lysine are summarized in Table V. Although methionine could not be determined because of its rather low level and the limited size of all samples from both tissues, the high lysine concentration in blood from rats fed polylysine or polymethionine covalently attached to polylysine was obviously consistent with an efficient hydrolysis of the polymers in the intestine and subsequent absorption of the free amino acids. By contrast, the levels of most other amino acids were either unchanged or only slightly affected. Thus, these data provided other evidence for the good availability of both polylysine and polylysine partly acylated by polymethionine side chains.

DISCUSSION

Polymerization of L-methionine on about half the ε-amino groups of poly-L-lysine allowed us to increase by more than 4-fold the size of the resulting branched-chain polymer in spite of the fact that its solubility was considerably decreased when the methionine to lysine molar ratio was beyond 1.5–2.0 (result not shown). As already observed with proteins (Gaertner and Puigserver, 1984a), it may not be possible to synthesize a water-soluble branched-chain polylysine containing covalently attached methionine polymers of an average chain length of more than seven to eight residues. Water-insoluble multichain polymers in which all the lysyl residues are acylated have also been obtained by Sela et al. (1955) by using a molar ratio of *N*-carboxyalanine anhydride to amino groups of 20–50 at pH 7.0. Thus, beyond a certain extent of polymerization, the nutritional value of methionine, a rather hydrophobic essential amino acid, is certainly limited. In the present study, polymethionine covalently attached to polylysine was nevertheless found to account for as much as 50% of the resulting multichain polymer weight without any important decrease in water solubility.

It is noteworthy that efficient hydrolysis of multioligo(L-methionyl)poly-L-lysine by proteases of the digestive tract, so long as covalently attached methionine did not exceed 60% of the polymer weight and less than half the ε-NH_2 groups were acylated, could be considered as a serious indication of good bioavailability of the polymer in vivo. The pattern of release of both amino acids from the multichain polymer was similar indeed to that obtained in the case of poly(L-methionyl)casein (Gaertner and Puigserver, 1986) under similar enzyme digestion conditions or in that of whole egg proteins as a result of pepsin–pancreatin digestion (Stahmann and Woldegiorgis, 1975). The use of a rather low protein diet containing gluten and gelatin, which are deficient in lysine and methionine, respectively, and supplemented with the branched-chain polymer confirmed this observation. The PER response of rats fed the basal protein diet supplemented with multioligo(L-methionyl)poly-L-lysine was not significantly different from that supplemented with the same amount of free L-methionine and free L-lysine. The observed increase in blood levels of free lysine in rats fed the basal diet supplemented with poly-L-lysine and free L-methionine or with multioligo(L-methionyl)poly-L-lysine was convincing evidence for the high biological availability of lysine from these polymers. The even more pronounced increase in muscular tissue levels of free lysine was actually consistent with already reported findings derived from experiments in which the requirement for this amino acid was widely fulfilled (Pawlack and Pion, 1968). On the other hand, a slight change in the amount of available dietary lysine in this concentration range can also result in a sharp decrease in muscle lysine concentration (Table V).

Since 30–40% of the ε-NH_2 groups of multioligo(L-methionyl)poly-L-lysine were protected by methionyl residues, it is worth stressing that the amount of unmod-

ified lysine in the diet was clearly below the amino acid requirement for growing rats. Consequently, lysine and methionine would be limiting if oligomethionine side chains as well as the polylysine moiety could not be hydrolyzed in vivo. Our feeding studies indicated that the efficiency of poly-L-lysine supplemented with free L-methionine was quite comparable to that of multioligo(L-methionyl)poly-L-lysine. They also further confirmed previous findings from in vitro enzyme hydrolysis of the model isopeptides N^{ϵ}-oligo(L-methionyl)-L-lysine and of poly(L-methionyl)proteins (Gaertner and Puigserver, 1984b,1986; Puigserver et al., 1979). Due to the fact that intestinal aminopeptidase is the enzymatic activity responsible for isopeptide bond hydrolysis and that it is almost completely deprived of activity on large polypeptides, covalently attached polymethionine side chains should first be hydrolyzed by gastric and pancreatic endopeptidases. By contrast, aminopeptidase-catalyzed hydrolysis of the isopeptide bond is a prerequisite for tryptic hydrolysis of lysyl bonds involving N^{ϵ}-protected amino groups. Finally, another point of interest was relevant to nutritional efficiency of poly-L-lysine for the young growing rat, confirming preliminary results obtained by Newman et al. (1980) with groups of two animals each over a 9-day period.

Poly-L-lysine and multioligo(L-methionyl)poly-L-lysine may therefore be used in formulating food and feed systems for improving their nutritional properties. The branched-chain polymer has also been shown to be quite resistant to rumen degradation (Bercovici et al., 1987).

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