Markwell, M. A. K.; Haas, S. M.; Bieber, L. L.; Tolbert, N. E. *Anal. Biochem.* **1978**, *87*, 206.
Matsuda, H.; Hirayama, O. *Bull. Fac. Agric., Shimane Univ.* **1979**, *13*, 105.
Pun, W. H.; Khan, A. A.; Chung, I.; Haydar, M.; Hadziyev, D. *Potato Res.* **1980**, *23*, 57.
Shepard, D. V.; Pitt, D. *Phytochemistry* **1976**, *15*, 1471.
Theologis, A.; Laties, G. G. *Plant Physiol.* **1980**, *66*, 890-896.
Walcott, P. J.; Kenrick, J. R.; Bishop, D. G. In "Biochemistry and Metabolism of Plant Lipids"; Wintermans, J. F. G. M.; Kuiper, P. J. C., Eds.; Elsevier: Amsterdam, 1982; p 297.
Wardale, D. A. *Phytochemistry* **1980**, *19*, 173.

Received for review June 8, 1984. Accepted October 2, 1984. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Attachment of *N*-Acetyl-L-methionine into Whole Soybeans and the Nutritional Consequences for the Rat

Günter Matheis, Lourminia C. Sen, Andrew J. Clifford, and John R. Whitaker*

The reactive *N*-hydroxysuccinimide ester of *N*-acetyl-L-methionine was used to attach *N*-acetyl-L-methionine (NAM) into whole soybeans. The modified soybeans contained 70% more methionine than the original soybeans. The added methionine was presumably present in the modified soybeans as NAM covalently attached to protein amino groups or other nucleophilic groups or tightly adsorbed by some noncovalent linkage since it was not removed by exhaustive dialysis. Rat feeding experiments using 10% protein diets demonstrated that NAM in the modified soybeans was biologically available, although to a lesser extent than free DL-methionine added to unmodified soybeans. The unadjusted protein efficiency ratio (PER) value of modified soybeans was 1.75 ± 0.10 compared to 1.19 ± 0.08 of control soybeans. When control soybeans were supplemented with free DL-methionine to equal the level of total methionine of modified soybeans, the PER value was increased to 2.29 ± 0.05. Part of the *N*,*N*-dimethylformamide (DMF) used to keep the *N*-hydroxysuccinimide ester of NAM soluble during the infusion procedure remained in the modified soybeans after dialysis. DMF caused a small transitory decreased growth rate of the rats during the initial 4 days.

The nutritive value of a food protein depends largely upon its ability to supply nutritionally essential amino acids that are absorbed by the gastrointestinal tract. Proteins from plants, single cells, and other less conventional sources are often of limited nutritional value because of their low content of one or more essential amino acids. As a consequence, a number of important studies have been done on food proteins to improve their nutritional value.

Supplementing foods with free essential amino acids is a common method, but it has some disadvantages (Puigserver et al., 1982). Among the disadvantages are possible losses during processing and cooking, Strecker degradation products that affect food flavor and color, and differences in the absorption rates and levels of amino acids present in proteins from that of free amino acids. The potential for improving protein quality by genetic means is promising, and seed protein quality has been improved by genetic and breeding practices (Mertz et al., 1964; Munck, 1972; Johnson and Mattern, 1978). Supplementation of cereal proteins with oilseed proteins (Sarwar et al., 1978) has also attracted considerable attention.

A variety of chemical and enzymatic methods have been applied to food proteins, or suggested for use, to improve their functional and nutritional properties (Feeney and Whitaker, 1977, 1982). Enzymatic protein degradation and resynthesis (plastein reaction) has been used, among other purposes, to incorporate essential amino acids into proteins (Fujimaki et al., 1977; Monti and Jost, 1979). Chemical methods available for covalent attachment of amino acids into proteins, via isopeptide bonds, include methods to modify carboxyl and amino groups of proteins. Limiting essential amino acids have been covalently attached to soy protein (Voutsinas and Nakai, 1979) and wheat gluten (Li-Chan et al., 1979) by enzymatic methods. A variety of amino acids have been incorporated into β-lactoglobulin (Puigserver et al., 1982) and casein (Puigserver et al., 1978, 1979a-c, 1982) by chemical methods.

Except for limiting amounts of methionine and cysteine, soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) are excellent sources of food protein (Jaffé, 1949; Kellor, 1974). Recently, the nutritional improvement of whole common bean seeds by methionine infusion has been reported (Antunes et al., 1979). However, during soaking (prior to cooking) about one-third of the infused methionine diffused out into the soaking water. Therefore, it seems desirable to bind the infused methionine strongly to the bean constituents to avoid losses during processing and cooking.

The purpose of the present work was to study the feasibility of infusing whole soybeans with the *N*-hydroxysuccinimide ester of NAM in order to covalently attach NAM to the amino groups of soybean protein and possibly other soybean constituents by the active ester method (Anderson et al., 1964). The nutritional value of soybeans modified in this way was studied in rats.

MATERIALS AND METHODS

Materials. Soybeans (No. 3585, Asgrow Seed Co., Sacramento, CA) were obtained from Professor B. S. Luh, Department of Food Science and Technology, University

Department of Food Science and Technology (G.M., L.C.S., and J.R.W.) and Department of Nutrition (A.J.C.), University of California, Davis, California 95616.

0021-8561/85/1433-0039$01.50/0

Table I. Proximate Composition of Soybean Flours[a]

	original soybean flour, %	control soybean flour, %	modified soybean flour, %
protein[b]	41.4	40.2	43.0
lipids	21.5	23.3	21.4
moisture	6.9	7.5	8.0
carbohydrates and ash[c]	30.2	29.0	27.6

[a] On a wet weight basis. [b] $N \times 6.25$. [c] By difference.

of California, Davis. *N*-Acetyl-L-methionine and sulfosalicylic acid were from Sigma Chemical Co., St. Louis, MO. *N*-Hydroxysuccinimide and dicyclohexylcarbodiimide were from ICN Pharmaceuticals, Irvine, CA, and Aldrich Chemical Company, Milwaukee, WI, respectively. The cereal-based stock diet was obtained from Ralston Purina, St. Louis, MO. Casein was from Sheffield Products, Memphis, TN, and DL-methionine from ICN Nutritional Biochemicals, Cleveland, OH. Glucose (cerelose) and corn oil (Mazola) were from Corn Products International, Englewood Cliffs, NJ. *N*,*N*-Dimethylformamide, diethyl ether, and methanol were from Mallinckrodt, St. Louis, MO. All other reagents and chemicals were of analytical grade.

Infusion and Attachment of *N*-Hydroxysuccinimide Ester of *N*-Acetyl-L-methionine into Whole Soybeans. *N*-Acetyl-L-methionine (NAM) was esterified with *N*-hydroxysuccinimide as described previously (Puigserver et al., 1979c). One kilogram of dry soybeans was presoaked in 1000 mL of 0.1 M sodium borate buffer, pH 9.0, containing 10% *N*,*N*-dimethylformamide (DMF) at 50 °C for 1 h. Ten grams of *N*-hydroxysuccinimide ester of NAM (NAM ester), dissolved in 200 mL of 0.1 M sodium borate buffer, pH 9.0, containing 10% DMF, was added, followed by the addition of 200 mL of 0.1 M sodium borate buffer, pH 9.0, containing 10% DMF. After 90 min, another 10 g of NAM ester, dissolved as above, was added. Then, 10 g of solid NAM ester was added every 15 min for 1 h. Thus, the total NAM ester added was 60 g. The total infusion time was 3 h at 50 °C. During infusion, the soybeans were gently stirred occasionally by hand, using gloves. The pH was kept constant at 9.0 by adding 2 N NaOH as required.

When the infusion was completed, the soybeans were washed 3 times with H_2O, placed in dialysis bags, and dialyzed for 30 h against H_2O (three changes). After dialysis, the soybeans were dried in a forced air dehydrator (Department of Food Science and Technology, UC Davis) at 55 °C for 8 h. Control soybeans (used in diets D_2 and D_4) were prepared in the same manner but in the absence of NAM ester. One batch of control soybeans (used in diet D_4, see below) was dialyzed 30 h against running deionized water.

Preparation and Chemical Analysis of Soybean Flours. Full-fat soybean flours were prepared by grinding hulled beans in a hammer mill (Mikro-Samplmill, Mikropul, Summit, NJ) to pass through a perforated plate with 1-mm holes.

The moisture content was determined by drying full-fat soybean flour samples to a constant weight in a vacuum oven at 80 °C for 74 h.

For protein determination, samples of soybeans flours were defatted (Schweiger and Muller, 1973) and digested by the method of Lang (1958), and the ammonia content was determined by nesslerization using the procedure of Johnson (1941). A standard curve was prepared by using previously dried ammonium sulfate.

The amino acid composition of soybean flours was determined with a Technicon Autoanalyzer (Tarrytown, NY) following hydrolysis of samples of defatted soybean flour with 6 N HCl in sealed tubes at 100 °C for 22 h (Spackman et al., 1958).

Table II. Essential Amino Acid Composition of Soybean Flours[a]

amino acid	g of amino acid/16 g of N: original soybean flour	control soybean flour	modified soybean flour	mg of amino acid/g of total essential amino acids: original soybean flour	modified soybean flour
Ile	5.0	5.5	5.0		
Leu	7.3	8.1	7.8		
Lys	6.2	6.8	6.3		
Phe	4.9	5.4	5.3		
Tyr	3.6	3.9	3.9		
Cys	1.2	1.2	1.0	30	23
Met	1.4	1.5	2.4	35	56
Thr	4.1	4.6	4.4		
Trp	n.d.[b]	n.d.[b]	n.d.[b]		
Val	5.3	6.1	5.6		
total S-containing amino acids	2.6	2.7	3.4	65	79

[a] Determined on defatted soybean flours. [b] Not determined. The Trp content given by Liener (1972) was used to calculate the sum of essential amino acids.

Table III. Percentage Composition of Soybean Diets (D_1–D_4)

original (D_1), modified (D_3), or control (D_2 and D_4) soybeans[a]	24.4[b]
added DL-methionine	0.1[c]
glucose	62.9[d]
corn oil	4.6[e]
mineral mixture[f]	6.0
vitamin mixture[f]	2.0

[a] Full-fat soybeans. [b] Corresponds to 10% soybean protein in the diets. [c] In diet D_4, 0.2% of DL-methionine was added at the expense of glucose. [d] 70.9% carbohydrate in the diet. [e] 10% lipids in the diet. [f] For the composition of mineral and vitamin mixtures, see Lee et al. (1978).

The proximate composition and the essential amino acid composition of the soybean flours are shown in Tables I and II, respectively.

Determination of *N*,*N*-Dimethylformamide (DMF). Diets were extracted with methanol or diethyl ether; blood plasma and urine were extracted with diethyl ether. The extracts were analyzed for DMF in a Varian Model 3700 gas chromatograph (Walnut Creek, CA) equipped with a methyl silicone capillary column (DB1, No. 09125, J and W Scientific, Rancho Cordova, CA). The initial temperature of the column was 65 °C for 1 min, followed by a programmed increase of the temperature (10 °C/min) until the final temperature of 170 °C was reached. Calibration curves were made with known amounts of DMF (1–10 μg) dissolved in methanol or diethyl ether.

Nutritional Evaluation of Modified and Unmodified Soybeans. Before feeding to rats, the soybeans were cooked for 45 min in a pressure cooker at 120 °C and 15 psi. The cooked beans were lyophilized and ground in a hammer mill (Mikro-Samplmill, Mikropul, Summit, NJ) to pass through a perforated plate with 3-mm holes.

All feeding studies were done with 21-day-old Sprague-Dawley male rats housed individually in suspended stainless steel cages. Throughout the study the rats had free access to food and water. In experiment 1, the rats were fed a cereal-based stock diet from Ralston Purina for 3 days, then divided into groups of six rats of approximately equal mean weight (65.8 ± 0.5 g), and as-

Table IV. Methionine, Cysteine/Cystine, Total S-Containing Amino Acids, and DMF Content of the Diets[a]

diet description	diet no.	Met, %	Cys/cystine, %	total S, %	DMF, %
original soybean (+0.1% Met)	D_1	0.24	0.12	0.36	0
control soybean 1 (+0.1% Met)	D_2	0.24	0.12	0.36	0.11
modified soybean (+0.1% Met)	D_3	0.34	0.12	0.46	0.07
control soybean 2 (+0.2% Met)	D_4	0.34	0.12	0.46	0.04
casein	D_5	0.30[b]	0.05[b]	0.35	0
casein (+0.2% DMF)	D_6	0.30[a]	0.05[a]	0.35	0.20

[a] Calculated from values of Table II. [b] Taken from Sarwar et al. (1983).

Table V. Percentage Composition of Casein Diets (D_5 and D_6)

casein	10.0
glucose[a]	72.0
corn oil	10.0
mineral mixture[b]	6.0
vitamin mixture[b]	2.0

[a] Diet D_6 contained 0.2% *N,N*-dimethylformamide at the expense of glucose. [b] For the composition of mineral and vitamin mixtures, see Lee et al. (1978).

signed at random to the soybean diets D_1–D_4 or the casein diet D_5 for a 27-day test period. The composition of diets D_1–D_4 is shown in Tables III and IV; the composition of diet D_5 is shown in Tables IV and V. Body weight and food intake were recorded 2 or 3 times weekly throughout the test period.

In experiment 2, casein diet D_6 (same as diet D_5 but containing 0.2% *N,N*-dimethylformamide; Table V) was fed to rats that had been fed the stock diet for 9 days and whose mean weight was 104.3 ± 0.8 g. Body weight and food intake were recorded 2 or 3 times weekly throughout the test period (21 days).

The nutritive values (protein efficiency ratios, PER) were calculated as the weight gained per unit weight of protein consumed.

Urine Sampling. Urine was collected during the last 4 days of each feeding trial. The urine within each dietary treatment was pooled and kept frozen (−20 °C) until analyzed for DMF.

Tissue Collection and Evaluation. The experiments were terminated at 9:00 a.m. on the last day of the feeding period. The rats were anesthetized with diethyl ether and bled by heart puncture into tubes containing 1.2% EDTA in 0.9% NaCl and evaluated for hematocrit. The blood was then centrifuged at 3000*g* at 5 °C for 20 min and the plasma isolated. Approximately one-tenth of the plasma volume from all rats within each dietary treatment was pooled to give six pooled plasma samples. The plasma samples were deproteinized with equal volumes of 6% sulfosalicylic acid and kept frozen (−20 °C) until analyzed (within 2 months) for free amino acids and DMF. The lithium citrate buffers for physiological fluid analysis were used for analysis of free amino acids ("Durrum Pico Buffer System IV Instruction Manual", 1973).

The kidneys, livers, and spleens were quickly excised from the anesthetized rats, immediately frozen (freeze clamped), weighed, and stored at −20 °C. The rats were then killed by a diethyl ether overdose.

Statistical Analysis. Data presented in Tables VI–VIII were subjected to analysis of variance. When *F* values were significant ($P < 0.05$), treatment means were analyzed by using the least significant difference method (Snedecor and Cochran, 1967).

Table VI. Average Weight Gain, Food Intake, and PER Values of Rats Fed Diets Containing 10% Protein[a]

diet description	diet no.	weight gain, g	food intake, g	unadjusted PER
experiment 1[b]				
original soybean (+0.1% Met)	D_1	67.3 ± 4.6[c]	302 ± 26[b]	2.23 ± 0.09[c]
modified soybean (+0.1% Met)	D_3	41.8 ± 4.0[b]	239 ± 9[a]	1.75 ± 0.10[b]
control soybean 2 (+0.2% Met)	D_4	58.5 ± 2.7[c]	255 ± 9[ab]	2.29 ± 0.05[c]
control soybean 1 (+0.1% Met)	D_2	27.2 ± 2.1[a]	228 ± 7[a]	1.19 ± 0.08[a]
casein	D_5	81.5 ± 4.1[d]	297 ± 12[b]	2.74 ± 0.06[d]
experiment 2[b]				
casein (+0.2% DMF)	D_6	61.7 ± 4.0	266 ± 8	2.32 ± 0.09

[a] Mean values of six rats ± SEM. Values within a column in experiment 1 not sharing a common superscript letter differ significantly ($P < 0.05$). [b] Feeding periods were 27 and 21 days for experiments 1 and 2, respectively. Average initial weights of rats were 65.8 ± 0.5 and 104.3 ± 0.8 g for experiments 1 and 2, respectively.

Table VII. Recalculated PER Values, Based on Weight Gain and Protein Consumption after Initial Delay in Growth[a]

diet no.	weight gain, g	food intake, g	recalculated PER
experiment 1			
D_1	48.5 ± 3.3[c]	192 ± 16[d]	2.58 ± 0.20[c]
D_3	29.3 ± 3.0[b]	152 ± 6[b]	1.90 ± 0.12[b]
D_4	43.8 ± 3.1[c]	167 ± 7[c]	2.61 ± 0.11[c]
D_2	22.2 ± 1.4[a]	138 ± 5[a]	1.62 ± 0.13[a]
casein (D_5)	60.3 ± 1.8[d]	197 ± 9[d]	3.07 ± 0.08[d]
experiment 2			
casein (D_6)	40.2 ± 2.2[c]	132 ± 4[a]	3.04 ± 0.10[d]

[a] As in Table VI, except weight gain, food intake, and recalculated PER are from days 11–27 for diets D_1–D_5 and from days 12–20 for diet D_6.

RESULTS

Analysis of Soybean Flours. The proximate composition and the essential amino acid composition of original, control, and modified soybean flours are shown in Tables I and II, respectively. The results are in agreement with published data (Wolf and Cowan, 1971; Liener, 1972).

The modified soybean flour contained 70% more methionine than the original flour. The added methionine was present in the soybeans as *N*-acetyl-L-methionine (NAM), since the *N*-hydroxysuccinimide ester of NAM is expected to be hydrolyzed completely during the infusion procedure. Whether the NAM was covalently bound to protein amino groups or other nucleophilic groups or whether it was tightly adsorbed by some noncovalent linkage is not known.

Growth. In the first feeding trial (experiment 1), four soybean diets (D_1–D_4, Table III) and one casein diet (D_5, Table V) were fed to rats, of average initial weights of 65.8 ± 0.5 g, for 27 days. All soybean diets were supplemented with DL-methionine (see Discussion). The sulfur amino acid content of the diets is listed in Table IV.

As shown in Figure 1, normal growth curves were obtained for the original soybean diet D_1 and casein diet D_5. In the case of control diets D_2 and D_4 and modified soybean diet D_3, however, a small transitory reduced growth rate was observed during the initial 4 days. The rats on diets D_2, D_3, and D_4 initially consumed less food than those on diets D_1 and D_5. Diets D_2, D_3 and D_4 were found to

Table VIII. Weights of Livers, Kidneys, and Spleens and Percentage of Red Blood Cells of Rats Fed Diets Containing 10% Protein[a]

diet description	diet no.	g/100 g of body weight			red blood cells, % of blood
		liver	kidneys	spleen	
experiment 1[b]					
casein	D_5	4.20 ± 0.06[a]	0.81 ± 0.01[a]	0.25 ± 0.01	42.8 ± 1.1[a]
original soybean (+0.1% Met)	D_1	4.34 ± 0.05[a]	0.82 ± 0.05[a]	n.d.[c]	41.8 ± 0.5[a]
modified soybean (+0.1% Met)	D_3	4.96 ± 0.20[b]	0.98 ± 0.03[b]	0.27 ± 0.02[d]	45.5 ± 0.9[b]
control soybean 2 (+0.2% Met)	D_4	5.27 ± 0.18[b]	0.87 ± 0.02[a]	0.25 ± 0.03	41.8 ± 0.2[a]
control soybean 1 (+0.1% Met)	D_2	4.32 ± 0.19[a]	1.04 ± 0.04[b]	n.d.[c]	47.5 ± 0.9[b]
experiment 2[b]					
casein (+0.2% DMF)	D_6	4.24 ± 0.15	0.79 ± 0.02	0.25 ± 0.01	42.8 ± 0.5

[a] Mean values of six rats ± SEM. Values within a column in experiment 1 not sharing a common superscript letter differ significantly ($P < 0.05$). [b] Feeding periods were 27 and 21 days for experiments 1 and 2, respectively. Average initial weights of rats were 65.8 ± 0.5 and 104.3 ± 0.8 g for experiments 1 and 2, respectively. [c] Not determined. [d] Mean value of four rats ± SEM.

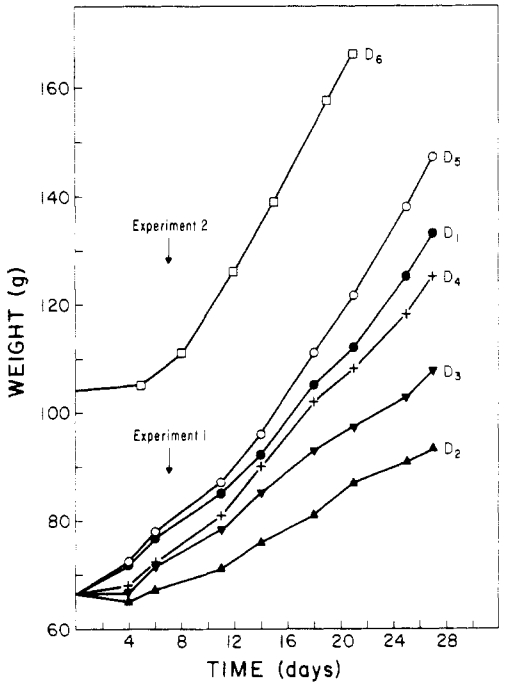

Figure 1. Growth curves of rats fed diets containing 10% protein. Data are mean values of six rats. Average initial weights of rats were 65.8 ± 0.5 and 104.3 ± 0.8 g for experiments 1 and 2, respectively. For diet descriptions (D_1–D_6), see Tables III–V.

have a peculiar taste to the investigators, similar to that of dimethylformamide (DMF). *N,N*-Dimethylformamide (DMF) was used in the infusion procedure to keep the *N*-hydroxysuccinimide ester of NAM soluble (see Materials and Methods). DMF was found in diets D_2, D_3, and D_4 (Table IV).

In a second feeding trial (experiment 2), DMF was added to a casein diet (D_6, Table V). When diet D_6 was fed to rats of average initial weights of 104.3 ± 0.8 g for 21 days, an initial delay in growth rate similar to that observed for diets D_2, D_3, and D_4 was found (Figure 1). While the growth rates following the period of delayed growth are not strictly comparable because the rats on diet D_6 were older, nevertheless the two sets of rats (diets D_5 and D_6) gained weight at the same rate after the initial delay in growth.

The data for body weight gain, food consumption, and protein efficiency ratios (PER) are summarized in Table VI. The lowest PER (53% that of the original soybeans) was found for control soybean 1 diet D_2. Although modified soybean diet D_3 and control soybean 2 diet D_4 contained ~42% more sulfur-containing amino acids (Table IV) than did the original soybean diet D_1, the PER of diet D_3 was less (77%) and the PER of diet D_4 was equal to that of diet D_1. The overall feed consumption of rats fed diets D_2, D_3, and D_4 was 75–84% of the feed consumption of rats fed diet D_1. Most of this decreased consumption of feed occurred within the first 4–5 days of the experiment. The reasons for smaller PER values of diets D_2 and D_3 compared to diet D_1 and the similar PER's of diets D_4 and D_1 are probably due to the reduced feed intake because of DMF. The PER of the DMF-containing casein diet D_6 in experiment 2 was also lower than that of the control diet D_5.

The PER values discussed in the preceding paragraph are based on overall weight gain and overall protein consumption. Comparison of the results obtained between day 11 and day 27 (day 12 and day 20 for diet D_6), following the period of delayed growth due to DMF, is shown in Table VII. These results show that the recalculated PER of the modified soybean diet D_3 was 74% of the original soybean diet D_1, 117% of the control soybean 1 D_2 diet, and 73% of control soybean 2 diet D_4. The recalculated PER of the casein diet D_6 with 0.2% DMF added was identical with the control casein diet D_5. Comparison of the recalculated PER value for diet D_2 (with DMF) with diet D_1 (no DMF) suggests that DMF continued to have an influence on growth beyond the initial delay in growth. The recalculated PER values for diets D_4 and D_1 were identical. The recalculated PER values of casein diet D_6 (with DMF) and diet D_5 (without DMF) after the initial delay in growth rate were identical. The rats on diet D_6 gained weight as fast as those on diet D_5, even though they were older and larger rats.

The growth data (Figure 1; Table VI) indicate that the NAM in the infused soybean diet D_3 was biologically available to the rat, although to a lesser extent (73%, based on recalculated PER in Table VII) than free DL-methionine added to the control 2 diet D_4.

Organ Weights. The weights of livers, kidneys, and spleens are shown in Table VIII. Rats fed modified soybean diet D_3 and control soybean 2 diet D_4 showed significantly higher weights of livers than rats fed the original soybean diet D_1, control soybean 1 diet D_2, and casein diet D_5. Slightly different results were obtained for the kidney weights. Rats fed modified soybean diet D_3 and control soybean 1 diet D_2 showed significantly higher weights of

Table IX. Plasma-Free Amino Acid Concentration of Rats Fed Diets Containing 10% Protein[a]

amino acids, μmol/100 mL of plasma	soybean diets				casein diets	
	D_1	D_2	D_3	D_4	D_5	D_6
O-phosphoserine	1	2	2	1	1	1
taurine	9	10	10	9	8	8
aspartic acid	4	3	4	3	4	3
hydroxyproline	4	0	0	2	4	4
threonine	14	23	23	13	53	49
serine	51	59	59	43	54	57
asparagine	11	8	11	7	6	13
glutamic acid	21	20	20	16	19	17
glutamine	75	77	81	70	74	78
proline	26	18	21	21	50	52
glycine	49	18	25	23	32	29
alanine	83	95	80	76	78	83
citrulline	12	9	11	11	12	11
valine	17	15	19	14	25	27
half-cystine	1	1	1	1	tr[b]	tr
methionine	6	5	6	7	6	6
isoleucine	11	6	8	7	9	10
leucine	14	11	15	11	18	21
tyrosine	12	7	9	10	19	19
phenylalanine	7	5	6	5	7	7
tryptophan	11	10	11	9	11	11
ornithine	31	43	40	33	12	13
lysine	38	48	42	45	80	80
histidine	9	10	11	8	11	11
arginine	15	5	10	14	15	16

[a] Values represent single determination on samples pooled from groups of six rats. Feeding periods were 27 and 21 days for experiment 1 (D_1–D_5) and experiment 2 (D_6), respectively. Average initial weights of rats were 65.8 ± 0.5 and 104.3 ± 0.8 g for experiment 1 (D_1–D_5) and experiment 2 (D_6), respectively. Blood samples were taken by cardiac puncture into tubes containing EDTA. [b] tr = trace.

kidneys than rats fed the original soybean diet D_1, control soybean 2 diet D_4, and casein diet D_5.

From experiment 2 it appears that a DMF-containing casein diet D_6 did not affect the organ weights (liver, kidney, and spleen) significantly.

Red Blood Cells. Blood hematocrit values (Table VIII) paralleled the results obtained for kidney weights. Hematocrit was significantly increased in rats fed control soybean 1 diet D_2 and modified soybean diet D_3.

Plasma-Free Amino Acid Patterns. The plasma free amino acid concentrations are shown in Table IX. Although modified soybean diet D_3 and control soybean 2 diet D_4 contained 70% more methionine than the original soybean diet D_1 and control soybean 1 diet D_2, this was not reflected in a higher methionine content of the plasma of rats fed diets D_3 and D_4. Most of the amino acid levels were either unchanged or only slightly affected. Different levels were found for hydroxyproline, threonine, glycine, and arginine. We have no explanation for these higher values, although they are still within physiologic ranges for rats.

The amino acid levels of plasma of rats fed casein diets D_5 and D_6 (D_6 contained DMF) were similar.

***N,N*-Dimethylformamide (DMF) in Blood Plasma and Urine.** Blood plasma and urine of rats fed the original soybeans (diet D_1; no DMF) and control soybean 1 (diet D_2; 0.11% DMF) were analyzed for DMF. Urine was collected during the last 4 days of each feeding trial. The urine within each dietary treatment was pooled and then analyzed for DMF. The pooled urine of rats fed diet D_2 contained 0.2 mg DMF/mL (=0.02%). No DMF was found in the urine of rats fed diet D_1.

Traces of DMF (~4 ng/mL) were found in the pooled plasma of rats fed diet D_2. No DMF could be detected in the plasma of rats fed diet D_1.

DISCUSSION

The soybeans infused with the *N*-hydroxysuccinimide ester of NAM contained 70% more methionine than the original soybeans. The active ester method (Andersen et al., 1964) used in the infusion procedure is known to covalently attach amino acids to ε-amino groups of protein-bound lysine (Puigserver et al., 1978, 1979a–c, 1982). No attempt was made in the present work to determine whether NAM is also attached to amino groups of proteins in the intact soybean. The possibilities remain that it may be linked to other nucleophilic groups present in the soybean or be tightly adsorbed by some noncovalent linkage.

The rat requires 600 mg of methionine/100 g of diet; one-third to half of this amount can be replaced by cystine (National Academy of Sciences, 1978). Since rats can use D- and L-methionine equally well, DL-methionine was therefore added to the soybean diets to provide 60% of the requirement for sulfur-containing amino acids. This level of supplementation also supplied the same level as that provided by casein diets (Table IV). Control soybean 2 diet D_4 differed from control soybean 1 diet D_2 in that it contained 0.2% (rather than 0.1%) of added free DL-methionine so as to have the same level of methionine as the modified soybean diet D_3 (Table IV). This permitted a comparison of the bioavailability of the bound NAM and free methionine in diets containing the same level of total methionine, subject to the effect of different levels of DMF remaining. The recalculated PER values (Table VII) permit a better comparison; they indicate that the bound NAM gave only 73% the PER value of an equivalent amount of free methionine.

DMF was used during the covalent attachment of amino acids to proteins in order to keep the *N*-hydroxysuccinimide esters of acyl amino acids in aqueous solution (Puigserver et al., 1978, 1979a–c, 1982). These investigators did not observe an effect of DMF on the growth rate of rats when the modified proteins were fed in diets at the 10% level. In the present work, where DMF was used for the same purpose, it caused an initial reduction in growth (Figure 1) and decreased the PER (Table VI) when modified soybeans, control soybeans, and a DMF-containing casein diet were fed at 10% protein level to rats. Organ weights and percentage of red blood cells were also affected (Table VIII). Analytical determinations indicated that DMF was not removed quantitatively from the intact soybeans by dialysis. Much of the initial effect of DMF on growth, and thus PER, was due to initial decreased food consumption. As shown in Table VII, the rats appear to adapt to the effect of DMF.

Modified soybeans and one batch of control soybeans (used in diet D_2) were dialyzed, in dialysis bags, for 30 h against three changes of water. These soybeans contained 0.7–1.1% of DMF (on a dry basis). One batch of control soybeans (used in diet D_4) was dialyzed for 30 h against running water. These soybeans contained only 0.4% of DMF (on a dry basis). Most DMF, if not all, can probably be removed from intact soybeans when they are dialyzed against running water or frequent changes of dialyzing water for a longer period of time (e.g., 3 days).

The toxicity of DMF is well established (Gosselin et al., 1976). The LD_{50} for rats is reported to be 1.4–3.8 g/kg (Massmann, 1956; Kutzsche, 1965), depending on the route of application. A reduction in growth rate in rats fed diets that contained 0.1% or 0.5% DMF was reported previously (Kutzsche, 1965). This effect was paralleled by reduced food intake and increased liver weight.

Our data show that the rats partly recovered from the transitory decrease in growth rate due to DMF after 4 days (Figure 1; Table VII). The presence of DMF in the urine and plasma of rats fed the control soybean 1 diet D_2 indicates that the rats did not metabolize (or at least did not metabolize all) the DMF. It has been reported previously that cats and rabbits also are unable to metabolize DMF (Massmann, 1956).

Despite the possible adverse effects of DMF, our data show that rats fed modified soybean diet D_3 grew better than rats fed control soybean 1 diet D_2 (Figure 1; Table VII) and the PER of diet D_3 was significantly higher than that of diet D_2 (Tables VI and VII). This indicates that some of the NAM in the modified soybeans was nutritionally available to the rat. NAM in the modified soybeans seems less available than free DL-methionine in control soybean 2 diet D_4 (Figure 1; Tables VI and VII). This, however, could also be the effect of the lower DMF content of diet D_4 compared to that of diet D_3 (Table IV).

Puigserver et al. (1978, 1979a–c, 1982) used the active ester method to prepare L-methionyl- and *N*-acetyl-L-methionylcaseins. These investigators found that in rat feeding studies, L-methionine covalently attached to casein was as readily available as the free amino acid; covalently bound NAM was also available but to a slightly lower extent.

The enzyme responsible for hydrolyzing the isopeptide bond in vivo was found to be the intestinal membrane-bound aminopeptidase (EC 3.4.11.2) (Puigserver et al., 1979a,b, 1982), although contributions of other enzymes cannot be ruled out. Acylase (EC 3.5.1.14) is responsible for the hydrolysis of NAM (Birnbaum et al., 1952; Endo, 1978). The nutritional and metabolic equivalency of free NAM and free methionine has been demonstrated with rats (Boggs et al., 1975; Rotruck and Boggs, 1975). The site of cleavage of NAM in vivo is not known (Stegink et al., 1980). NAM has been approved as a food additive recently ("Code of Federal Regulations", 1979).

The results presented here indicate that infusion of soybeans (and possibly other beans) with the *N*-hydroxysuccinimide ester of NAM is possible. However, care must be taken to remove solvents that may have an adverse effect on the animals.

ACKNOWLEDGMENT

We thank Prof. B. S. Luh, Department of Food Science and Technology, University of California, Davis, for the gift of the soybeans, Prof. Q. R. Rogers, School of Veterinary Medicine, University of California, Davis, for the analysis of plasma free amino acids, Prof. W. G. Jennings, Department of Food Science and Technology, University of California, Davis, for the use of the gas chromatograph, Virginia DuBowy for checking the references, and Clara Robison for typing the manuscript.

Registry No. NAM, 65-82-7; NAM *N*-hydroxysuccinimide ester, 93184-38-4.

LITERATURE CITED

Anderson, G. W.; Zimmermann, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1964**, *86*, 1839.
Antunes, P. L.; Sgarbieri, V. C.; Garruti, R. S. *J. Food Sci.* **1979**, *44*, 1302.
Birnbaum, S. M.; Levintow, L.; Kingsley, R. B.; Greenstein, J. P. *J. Biol. Chem.* **1952**, *194*, 455.
Boggs, R. W.; Rotruck, J. T.; Damico, R. A. *J. Nutr.* **1975**, *105*, 326.
"Code of Federal Regulations". FDA: Washington, DC, 1979; No. 172.372, p 21.
"Durrum Pico Buffer System IV Instruction Manual". Durrum Chemical Corp.: Palo Alto, CA, 1973; p 1.
Endo, Y. *FEBS Lett.* **1978**, *95*, 281.
Feeney, R. E.; Whitaker, J. R., Eds. "Food Proteins: Improvement Through Chemical and Enzymatic Modification"; American Chemical Society: Washington, DC, 1977; Adv. Chem. Ser. No. 160.
Feeney, R. E.; Whitaker, J. R.; Eds. "Modification of Proteins: Food, Nutritional, and Pharmacological Aspects"; American Chemical Society: Washington, DC, 1982; Adv. Chem. Ser. No. 198.
Fujimaki, M.; Arai, S.; Yamashita, M. American Chemical Society: Washington, DC, 1977; Adv. Chem. Ser. No. 160, p 156.
Gosselin, R. E.; Hodge, H. C.; Smith, R. P.; Gleason, M. N. "Clinical Toxicology of Commercial Products. Acute Poisoning", 4th ed.; Williams and Wilkins: Baltimore, MD, 1976; p 135.
Jaffé, W. G. *Proc. Soc. Exp. Biol. Med.* **1949**, *71*, 398.
Johnson, M. J. *J. Biol. Chem.* **1941**, *137*, 575.
Johnson, V. A.; Mattern, P. J. *Adv. Exp. Med. Biol.* **1978**, *105*, 301.
Kellor, R. L. *J. Am. Oil Chem. Soc.* **1974**, *51*, 77A.
Kutzsche, A. *Arzneim.*-Forsch. **1965**, *15*, 618.
Lang, C. A. *Anal. Chem.* **1958**, *30*, 1692.
Lee, H. S.; Sen, L. C.; Clifford, A. J.; Whitaker, J. R.; Feeney, R. E. *J. Nutr.* **1978**, *108*, 687.
Li-Chan, E.; Helbig, N.; Holbek, E.; Chau, S.; Nakai, S. *J. Agric. Food Chem.* **1979**, *27*, 877.
Liener, I. E. In "Soybeans: Chemistry and Technology. Vol. 1. Proteins"; Smith, A. K.; Circle, S. J., Eds.; Avi: Westport, CT, 1972; p 203.
Massmann, W. *Br. J. Ind. Med.* **1956**, *13*, 51.
Mertz, E. T.; Bates, L. S.; Nelson, O. E. *Science (Washington, D.C.)* **1964**, *145*, 279.
Monti, J. C.; Jost, R. *J. Agric. Food Chem.* **1979**, *27*, 1281.
Munck, L. *Hereditas* **1972**, *72*, 1.
National Academy of Sciences. "Nutrient Requirements of Laboratory Animals"; NAS: Washington, DC, 1978; p 7.
Puigserver, A. J.; Gaertner, H. F.; Sen, L. C.; Feeney, R. E.; Whitaker, J. R. "Modification of Proteins: Food, Nutritional, and Pharmacological Aspects"; American Chemical Society: Washington, DC, 1982; Adv. Chem. Ser. No. 198, p 149.
Puigserver, A. J., Sen, L. C.; Clifford, A. J.; Feeney, R. E.; Whitaker, J. R. *Adv. Exp. Med. Biol.* **1978**, *105*, 587.
Puigserver, A. J.; Sen, L. C.; Clifford, A. J.; Feeney, R. E.; Whitaker, J. R. *J. Agric. Food Chem.* **1979a**, *27*, 1286.
Puigserver, A. J.; Sen, L. C.; Feeney, R. E.; Whitaker, J. R. *Ann. Biol. Anim., Biochim., Biophys.* **1979b**, *19*, 749.
Puigserver, A. J.; Sen, L. C.; Gonzales-Flores, E.; Feeney, R. E.; Whitaker, J. R. *J. Agric. Food Chem.* **1979c**, *27*, 1098.
Rotruck, J. T.; Boggs, R. W. *J. Nutr.* **1975**, *105*, 331.
Sarwar, G.; Christensen, D. A.; Finlayson, A. J.; Friedman, M.; Hackler, L. R.; MacKenzie, S. L.; Pellett, P. L.; Tkachuk, R. *J. Food Sci.* **1983**, *48*, 526.
Sarwar, G.; Sosulski, F. W.; Bell, J. M.; Rowland, J. P. *Adv. Exp. Med. Biol.* **1978**, *105*, 415.
Schweiger, R. G.; Muller, S. A. U. S. Patent 3 714 210, 1973.
Snedecor, G. W.; Cochran, G. W. "Statistical Methods", 6th ed.; Iowa State University Press: Ames, IA, 1967; p 258.
Spackman, D. H.; Stein, W. H.; Moore, S. *Anal. Chem.* **1958**, *30*, 1190.
Stegink, L. D.; Filer, L. J., Jr.; Baker, G. L. *J. Nutr.* **1980**, *110*, 42.
Voutsinas, L. P.; Nakai, S. *J. Food Sci.* **1979**, *44*, 1205.
Wolf, W. J.; Cowan, J. C. "Soybeans as a Food Source"; CRC Press: Cleveland, OH, 1971.

Received for review October 7, 1983. Revised manuscript received August 27, 1984. Accepted September 18, 1984. Financial support was received from Grants MA 888/1-1 and -1-2 from the Deutsche Forschungsgemeinschaft (to G. M.) and from National Institutes of Health Grant AM 26031.