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Effect of Methionine on Nicotinic Acid and Indoleacetic Acid Pathways of Tryptophan Metabolism *in vivo*. (30342)

HERBERT SPRINCE, CLARENCE M. PARKER, DOROTHY JAMESON, AND JOSEPH A. JOSEPHS, JR.
U. S. Veterans Administration Hospital, Coatesville, Pa. and Department of Biochemistry, Graduate School of Medicine, University of Pennsylvania, Philadelphia

Previous clinical studies from this laboratory have revealed that an oral test load-dose of DL-methionine following oral intake of a monoamineoxidase inhibitor (tranylcypromine) markedly increased certain urinary indoles above those levels obtained with the monoamineoxidase inhibitor alone. The indoles in question were found to be tryptamine and the glucuronide conjugate of indoleacetic acid as measured by indoleacetamide(1). Tryptamine is known to be an intermediary metabolite of tryptophan metabolism capable of being oxidized to indoleacetic acid by monoamineoxidase in animal tissues as well as in intestinal bacteria(2). To investigate further the methionine effect *per se* on tryptophan metabolism, rats were studied under controlled dietary conditions using synthetic diets. It is the purpose of this paper to report the effect of excess methionine on tryptophan metabolism *in vivo* as measured by urinary N^1-methylnicotinamide (MNA) and indoleacetic acid (IAA), 2 metabolites originating from tryptophan, by different metabolic pathways(3). Measurement of urinary creatinine (CR) was also included in this study because of the involvement of methionine in its formation.

Materials and methods. Forty-eight Sprague-Dawley male rats, approximately 55 days old, weighing about 200 g, and maintained for 7 to 10 days prior to experimentation on a control 30% casein diet (CAS),* were divided into 6 groups of 8 rats per group. Each group was placed on an appropriate test diet. In some instances more than one group of 8 rats was run with a given diet. The test diets used were as follows: (1) diet (CAS)* = 30% casein control; (2) diet (CS) = complete-synthetic amino acid diet

* Composition of 30% casein diet (CAS) in g/100 g of diet was: casein, 30.0; corn starch, 46.0; yellow dextrin, 2.0; non-nutritive fiber (cellulose), 4.0; NRRL salt mix No. 446(4), 3.0; cottonseed oil, 14.0, and vitamin supplement in corn starch, 1.0. One gram of corn starch-vitamin supplement supplied the following vitamins in mg/100 g of diet: p-aminobenzoic acid, 11.02; ascorbic acid (coated 97.5% ascorbic acid), 101.75; biotin, 0.04; Ca pantothenate, 6.61; choline dihydrogen citrate, 371.52; folic acid, 0.20; i-inositol, 11.02; menadione, 4.96; nicotinic acid, 9.92; pyridoxine • HCl, 2.20; riboflavin, 2.20; thiamin • HCl, 2.20; vit. A (dry stabilized, 500,000 U.S.P. units/g), 3.97; vit B_{12} (0.1% in mannitol), 2.98; vit D_2 (dry, 500,000 U.S.P. units/g), 0.44; vit E acetate (25%, 250 I.U. vit E/g), 48.50; and corn starch q.s., 420.47. This diet was prepared by General Biochemicals.

described in detail in the next paragraph; (3) diet (CS-N) = same as (CS), but minus nicotinic acid or nicotinamide; (4) diet (CS-N-M) = same as (CS-N), but minus methionine; (5) diet (CS-N + 4% M) = same as (CS-N), but plus 4% methionine (total methionine content = 4.806%); (6) diet (CS-N-M-T) = same as (CS-N), but minus both methionine and tryptophan.

The exact composition of test diet (CS), from which the other test diets were derived, was as follows (g/100 g of diet): L-arginine·HCl, 1.209; L-histidine·HCl·H_2O, 0.484; L-isoleucine, 0.448; L-leucine, 0.717; L-lysine, 1.227; DL-methionine, 0.806; DL-phenylalanine, 0.806; DL-threonine, 0.896; DL-tryptophan, 0.358; DL-valine, 1.254; DL-alanine, 0.358; L-asparagine, 0.896; L-glutamic acid, 4.569; glycine, 1.792; DL-serine, 0.448; L-tyrosine, 0.896; cottonseed oil, 7.175; Wesson salt mix(5), 3.587; sodium bicarbonate, 1.525; sucrose, 59.677; non-nutritive fiber (cellulose), 8.780; and cod liver oil, 1.794. Vitamins added were (mg/100 g of diet): thiamine·HCl, 0.538; riboflavin, 0.538; pyridoxine·HCl, 0.538; calcium pantothenate, 2.687; nicotinic acid, 2.239; choline chloride, 179.2; biotin, 0.0449; folic acid, 0.179; i-inositol, 89.580; menadione, 0.448; vit B_{12} (0.1% in mannitol), 4.479; and α-tocopherol, 17.917. This diet was prepared by General Biochemicals (J. R. Thompson formulation).

On test diet (CS), 8 male rats with an initial weight of 200 g were found to show an approximate mean weight increase of 3.6 g/day over a 2-week test period. This compared favorably with 3.6 g/day over a 4-week test period calculated by interpolation from a theoretical rat growth curve by Warner(6). Although with casein diet (CAS), 200-g rats showed a mean weight increase of about 6.1 g/day over a 2-week test period, it should be noted that this casein diet contained more fat than the amino acid diet (CS). Casein diet (CAS) is in reality a *secondary* control serving as a rough guide for the effect of an optimal protein diet on CR, MNA, and IAA excretion. Actually, within the context of our experimental conditions, the *primary* control is diet (CS) which serves as the real basis of comparison for the effects observed.

During the test period, rats were maintained as follows. Rats were housed and fed in Hoeltge rat metabolism cages (one rat per cage). Daily records were kept of rat weight, food consumption, and urine volume for a period of 2 to 3 weeks. Twenty-four-hour urine samples were collected under toluene, pooled for each group, and stored in a deep-freeze (−15°C) at the beginning of the experiment (zero time) and after 2 weeks on the test diets. Urinary determinations were made for CR, MNA, and IAA. CR was assayed by the standard alkaline-picrate method(7). MNA was measured by a modification of the method of Sarett(8). This modification consisted of treatment with 0.5 N HCl (0.1 of total volume) at 40°C for 5 minutes in a glass stoppered tube, just prior to addition of benzidine reagent. IAA was determined both as free, unhydrolyzed IAA (FIAA) and total, acid-hydrolyzed IAA (TIAA). Despite certain limitations, acid hydrolysis rather than alkaline hydrolysis for total IAA was used for reasons set forth by Weissbach *et al*(2).

IAA was determined by a newly developed paper chromatographic method to avoid interference from a closely related chromatographic spot normally found in rat urine and tentatively identified by chromatographic R_f values in 3 different solvent systems(9) as indoleacetylglycine (IAGly) intermixed with small amounts of other indolic compounds. Urine (2-5 ml) unhydrolyzed, or hydrolyzed by acidification to 1 N with HCl and heating at 80°C for 15 minutes, was adjusted to pH 5-5.5, diluted to convenient volume (10-20 ml), saturated with $(NH_4)_2SO_4$, extracted with ethyl ether, the ether extract evaporated to dryness, and dissolved in 95% ethanol to convenient volume (2-5 ml). An aliquot of the ethanol extract (≈ approx. 0.5-1.5 mg urinary CR) was then subjected to 2-dimensional paper chromatography (*first solvent,* isopropanol/ammonium hydroxide/water, 80/10/20; *second solvent,* isopropanol/pyridine/aqueous 25% dimethylamine-glacial acetic acid mixture adjusted to pH 6.2/water, 75/10/5/10) and sprayed with Ehrlich benzalde-

hyde-nitrite reagent(10). Two blue spots resulted: one was the tentatively-identified spot mentioned above (R_f = 0.55, 0.28 respectively); the other was the IAA spot (R_f = 0.51, 0.64 respectively). The blue IAA spot was extracted into 2 ml alcoholic-HCl solution (1 part 3 N HCl + 1 part 95% ethanol) and the optical density read at 580 mμ. With this method, 5 μg IAA gave an optical density value of approximately 0.100 with a cuvette of 1.0 cm light-path. Optical density increased linearly with IAA over a range of 1-30 μg. Reagent blanks gave very low optical density values (0.01-0.02). Recovery tests of added IAA in FIAA assays ranged 90-100%; in TIAA assays, 75-90%. For rapid one-way chromatographic separations, good results have also been obtained with the second solvent system *per se*.

Results and discussion. Data are set forth in Table I.

Rat weight and CR excretion. Values of mean rat weight and mean CR excretion after 2 weeks were found to be as expected. In the case of mean rat weight, the synthetic diets (CS) and (CS-N) showed approximately the same weight increase (*ca* 20%), probably because the rat can synthesize nicotinic acid from dietary tryptophan. This increase was less than that observed with the casein (CAS) control diet (*ca* 40%). As a rule, weight gain is faster with an optimal protein diet than with an optimal synthetic amino-acid diet; moreover, in this case diet (CAS) contained more fat than diet (CS). Synthetic diets deficient in methionine alone (CS-N-M) and also in tryptophan (CS-N-M-T) showed about the same weight loss (*ca* 25%). The excess-methionine diet (CS-N + 4% M) prevented growth, confirming the toxic effect of excess methionine observed by others(11). In the case of mean CR excretion, synthetic diets (CS) and (CS-N) resulted in approximately the same increase in urinary CR output (*ca* 25%). On the other hand, a slight drop (*ca* 13%) occurred with the casein diet, probably reflecting nitrogen retention during a period of more rapid weight gain. CR excretion was decreased with both methionine-deficient diets (*ca* 15%) and increased with excess-methionine diet (*ca* 38%). These findings were undoubtedly related to the fact that methionine furnishes methyl groups for creatinine formation. Variations in CR output were relatively small and predictable compared to those of MNA and IAA.

MNA excretion. Data obtained for mean urinary MNA excretion after the 2-week interval demonstrated some interesting rela-

TABLE I. Effect of Excess-Dietary Methionine on Mean Excretion of Creatinine (CR), N¹-Methylnicotinamide (MNA), and Indoleacetic Acid (Total, TIAA; Free, FIAA) in the Urine of Rats on Complete-Synthetic Amino Acid Diets for a 2-Week Test Period.

Diet*	Total No. rats	Mean rat wt (g)			Mean urinary excretion per 24 hr per kg rat								
					CR (mg)			MNA (mg)			TIAA (μg)/FIAA (μg)		
		Weeks 0	Weeks 2	% Change	Weeks 0	Weeks 2	% Change	Weeks 0	Weeks 2	% Change	Weeks 0	Weeks 2	% Change
Casein (CAS)	16	201	287	+43	32	28	−13	16	12	−25	87/65	65/58	−25/−11
(CS)	8	207	257	+24	31	40	+29	14	14	0	157/150	376/214	+139/+43
(CS − N)	32	204	243	+19	30	37	+23	15	8	−47	74/60	216/90	+192/+50
(CS − N − M)	8	203	150	−26	30	27	−10	20	8	−60	126/76	154/33	+22/−57
(CS − N + 4% M)	24	203	200	−1	29	40	+38	18	1	−94	108/75	1160/231	+974/+208
(CS − N − M − T)	8	208	162	−22	33	28	−15	18	.3	−98	63/47	7/7	−89/−85

* For abbreviations, see text. Mean food consumption (g/24 hr) for above diets in descending order beginning with casein were: (a) at zero time 12.4, 13.1, 13.2, 14.0, 13.0; after 2 weeks 15.6, 15.1, 12.4, 6.6, 9.5, 7.4.

tionships. Diet (CS-N) showed an MNA decrease (*ca* 47%), but diet (CS) showed no change. In the former case, MNA probably originates primarily from dietary tryptophan; in the latter case, both dietary tryptophan and nicotinic acid contribute to MNA formation. With the casein diet, MNA was reduced (*ca* 25%), probably again reflecting nitrogen retention during more rapid weight gain. With methionine-deficient diet (CS-N-M), a further drop (*ca* 60%) occurred probably because of lack of methionine which is necessary for methylation of nicotinamide to MNA. Quite unexpected, however, was the marked decrease in MNA excretion (*ca* 94%) with excess-methionine diet (CS-N + 4% M). This drop was similar in magnitude to the drop (*ca* 98%) obtained with the diet deficient in both methionine and tryptophan (CS-N-M-T), a condition wherein MNA formation must necessarily be minimal. Although excess-dietary methionine has been reported to diminish MNA excretion(12), the significance of this observation has not been fully appreciated. The degree of diminution of urinary MNA suggests that the metabolic pathway of tryptophan to MNA thru nicotinamide may be inhibited at some intermediate point by an excess of methionine.

IAA excretion. Values of free and total IAA excretion after the 2-week interval revealed another striking situation. Concomitant with the marked decrease in MNA excretion, diet (CS-N + 4% M) effected a marked rise in both FIAA and TIAA. This rise in comparison with similar IAA values for diet (CS-N) as control was about 4- and 5-fold, respectively (*i.e.*, 208%/50% and 974%/192%). Thus, excess-dietary methionine not only decreases urinary MNA but also increases urinary IAA suggesting a shift in tryptophan metabolism from the nicotinic acid pathway to the IAA pathways(3).

Intraperitoneally-administered methionine. To examine the possible role of intestinal microflora, a group of 8 rats on diet (CS-N) were injected intraperitoneally (IP) with 5 ml of a 2.2% solution of methionine (adjusted to pH 7.2 with NaOH and calculated to be approximately isotonic with serum) and compared with another group of 8 rats on the same diet but injected similarly with 5 ml of physiological (0.9%) saline solution as a control. Injections were made daily for a period of one week. Urinary excretion patterns of MNA and IAA with excess-intraperitoneally-administered methionine showed the same trends as those obtained with excess-dietary methionine. Thus, the percent changes after one week for IP methionine *vs* IP saline control respectively were as follows: (a) a decrease in MNA of 63% *vs* 32% from initial (zero time) values of 16.4 *vs* 16.5 mg/24 hr/kg rat; (b) an increase in FIAA of 323% *vs* 174% from initial values of 65.0 *vs* 69.4 μg/24 hr/kg rat; and (c) an increase in TIAA of 404% *vs* 228% from initial values of 109.0 *vs* 116.2 μg/24 hr/kg rat. These observations favor tissue metabolism rather than intestinal microflora as the more likely explanation of the excess methionine effect. Experiments involving intestinal sterilization by anti-microbial agents (*e.g.*, antibiotics and sulfadrugs) were deemed to be less desirable than those with intraperitoneally-injected methionine because of possible inhibitory action of such agents on tissue metabolism.

Summary. In rats fed synthetic amino-acid test diets containing tryptophan but deficient in nicotinic acid or nicotinamide, an excess of dietary methionine lowers the urinary level of N^1-methylnicotinamide (MNA) and raises the urinary level of indoleacetic acid (IAA). The urinary creatinine (CR) level is only slightly increased. Excess methionine administered intraperitoneally to rats fed the same diet results in a similar urinary excretion pattern of MNA and IAA. As a working hypothesis, it is suggested that excess methionine may favor the channeling of tryptophan metabolism into indoleacetic acid production by inhibiting the nicotinic acid pathway or stimulating those pathways leading to formation of indoleacetic acid.

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Effect of Actinomycin-D on Calcium Homeostasis. (30343)

E. C. KHOO AND K. KOWALEWSKI
Surgical-Medical Research Institute, University of Alberta, Edmonton, Alberta, Canada

Actinomycin-D, an inhibitor of DNA dependent synthesis of RNA(1), has been shown to block the actions of several hormones(2,3). Recent studies(4,5) have also clearly established the fact that this drug abolishes the hypercalcemic response of parathyroidectomized rats to exogenous parathyroid hormone. The hypothesis has been advanced that Actinomycin-D, by blocking protein synthesis, antagonizes the action of parathyroid hormone on calcium mobilization(4). If this hypothesis is valid, then the drug should produce a hypocalcemic response when given to normal animals by blocking the action of endogenous parathyroid hormone.

The following experiments were performed to determine if such a hypocalcemic effect could be produced in rats with intact parathyroid glands.

Methods. Male Sprague-Dawley rats weighing from 150 to 170 g were used. Actinomycin-D (Merck, Sharp and Dohme) was injected intraperitoneally. Control rats were injected with an equal volume of normal saline. The following groups of animals were used: 1) Normal, injected with Actinomycin-D, in a dose of 1 μg/g body weight; 2) Nephrectomized rats: bilateral nephrectomy was performed in these animals 24 hours prior to injection of Actinomycin-D, 1 μg/g body weight; 3) Adrenalectomized rats: bilateral adrenalectomy was done 48 hours prior to injection of Actinomycin-D, 0.5 μg/g body weight. These rats were allowed 0.9% saline for drinking. 4) In the last group of rats total thyro-parathyroidectomy was performed under ether anesthesia. The parathyroid glands were then transplanted into the gastrocnemius muscle. A week later blood was obtained through the retro-orbital venous plexus and serum calcium determined. Only those rats with serum calcium above 7 mg% indicating functional parathyroids were injected with Actinomycin-D, 1 μg/g body weight.

In all experiments, after administration of the drug, the rats were given no food, only drinking water, until they were anesthetized at the appropriate time interval and bled through the abdominal aorta. Serum calcium determination was performed by the Kingsley-Robnett method(6).

Results. In a preliminary study adrenalectomized rats appeared particularly sensitive to Actinomycin-D and did not tolerate a dose of 1 μg/g body weight. None of them survived the 24-hour period after injection. A mortality of approximately 20% occurred, however, under the conditions of this experiment, when a smaller dose of 0.5 μg/g body weight was used. Other groups of rats tolerated well a dose of 1 μg/g body weight.

Table I shows the average serum calcium of various groups of Actinomycin-D treated and saline injected control rats killed 24 hours after injections. The mean serum cal-