PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **188**, 169–172 (1988)

Effects of Dietary Nonspecific Nitrogen on [^{14}C]Glutamate and [^{14}C]Proline Incorporation into Bone Proteins in Chicks[1] (42723)

J. C. ROGLER,* G. H. K. DE MORAES,† AND R. G. ELKIN*

**Department of Animal Sciences, Purdue University, West Lafayette, Indiana 47907, and †Departmento de Quimica, Universidade Fereral de Vicosa, 36570 Viçosa, Minas Gerais, Brazil*

Abstract. The incorporation of [^{14}C]glutamic acid into EDTA-soluble and -insoluble calvaria protein *in vitro* and [^{14}C]proline into EDTA-insoluble femur protein *in vivo* was determined in chicks fed inadequate and adequate levels of nonspecific nitrogen (glutamic acid). In each instance, the amount of amino acid incorporated into bone protein was reduced by the low level of nonspecific nitrogen. It was concluded that the high incidence of leg abnormalities observed in chicks fed purified diets containing adequate levels of indispensable amino acids but lacking in total nitrogen might be associated with an inability to form bone matrix protein.

A deficiency of total or "nonspecific" nitrogen in an otherwise adequate crystalline amino acid diet reduced growth rate and produced a high incidence of leg abnormalities in young chicks (1). The condition was corrected either by increasing the L-glutamic acid content of the diet or by adding an isonitrogenous amount of a mixture of dispensable L-amino acids. Supplemental diammonium citrate was also effective in overcoming the leg problems (2). The addition of seven D-amino acids to a similar crystalline amino acid diet deficient in nonspecific nitrogen drastically reduced growth rate, but the leg anomalies were still prevalent. The addition of L-glutamic acid corrected the abnormal leg condition without influencing the growth retardation caused by the D-amino acids (2).

The present study was undertaken to determine the effects of inadequate and adequate levels of dietary nonspecific nitrogen on the incorporation of ^{14}C-labeled amino acids into bone protein.

Materials and Methods. Day-old White Mountain male chicks were reared in electrically heated batteries with raised wire-mesh floors and provided water and the experimental diets *ad libitum*. The purified diet, described previously (1), contained adequate levels of all indispensable amino acids, vitamins, minerals, and essential fatty acids. Two levels of glutamic acid (5 and 12.5%) were used to provide inadequate and adequate levels of total or "nonspecific" nitrogen. Each of the two experimental diets was fed to four replicate pens of seven chicks each for the specified times.

At 7 days of age, all seven chicks in one replicate from each diet were bled by cardiac puncture using a heparinized needle and syringe. Each blood sample was transferred to a plastic centrifuge tube and placed on ice and the plasma was subsequently obtained by centrifugation at 10,000*g* for 10 min at 4°C. Equal aliquots of plasma from each chick within a treatment were pooled. Immediately after bleeding, the first six chicks from each treatment were killed by cervical dislocation and the calvaria removed and placed in cold saline. Three milliliters of cold plasma from chicks fed 5% glutamic acid and 100 μl of a saline solution containing 2 μCi of [^{14}C]glutamic acid (250 mCi/mmole) were placed in each of six 25-ml Erlenmeyer flasks maintained on ice. The calvaria from the same treatment were removed from the cold saline, blotted, and weighed. One calvarium was placed in each flask. Plasma and calvaria from chicks fed 12.5% glutamic acid were treated similarly. The unstoppered flasks were incubated with constant shaking for 4 hr in a water bath maintained at 37°C. At the end of the incubation period, each calvarium was removed, rinsed thoroughly with saline,

[1] Journal Paper Number 11,345, Purdue University Agricultural Experiment Station. This research was supported in part by a grant from UNESCO.

0037-9727/88 $1.50

and placed in another 25-ml Erlenmeyer flask containing 3 ml of 0.5 *M* disodium (ethylenedinitrilo) tetraacetic acid (EDTA). The EDTA was decanted after 24 hr and the calvaria were reextracted twice with 3 ml of EDTA for 24 hr each.

The EDTA extracts were combined (9 ml) and the proteins, including the γ-carboxyglutamic acid containing osteocalcin (3), were extracted using a modification of the phenol procedure of Landt and Butler (4). Modification consisted of extracting the proteins from the EDTA-soluble fraction with 4.5 ml of liquid phenol saturated with the 0.5 *M* EDTA solution. After thorough mixing, the two phases were allowed to separate and the phenol phase was collected and washed three times with 0.5 *M* EDTA to remove nonprotein radioactivity. One milliliter of the phenol fraction was pipetted into counting vials containing 20 ml of Dimilume-30 (Packard Instrument Co., Downers Grove, IL) and the samples were counted in a Packard Model 460C liquid scintillation counter. Background-subtracted sample counts were corrected for quenching by external standardization with a ^{226}Ra source.

Each EDTA-extracted calvarium was placed in a counting vial and 2 ml of Soluene-350 (Packard Instrument Co.) was added. The samples were allowed to sit overnight at room temperature and then heated for 2 to 4 hr in a water bath at 50°C until completely digested. The vials were allowed to cool to room temperature and 15 ml of Dimilume-30 was added to each digested calvarium. Radioactivity was determined as described for the phenol fractions.

Amino acid concentrations were determined in duplicate samples of ultrafiltered pooled plasma from each treatment using precolumn derivatization with phenylisothiocyanate and reversed-phase high-performance liquid chromatography (5). The values obtained for glutamic acid and proline were used to determine specific activity of the respective amino acids and incorporation into protein calculated on a mole basis.

On the day following removal of the calvaria (Day 8), two chicks from each of two replicates and three chicks from the remaining replicate of each treatment were selected at random, weighed, and injected intraperitoneally with [^{14}C]proline (250 mCi/mmole) in saline at a dose of 5 μCi/100 g of body wt. The chicks were killed by cervical dislocation 24 hr postinjection. Both femurs from each chick were removed, cleaned of adhering tissue, and split longitudinally and the marrow was removed by a stream of distilled water. Both halves of each femur were placed in a counting vial and demineralized with 15 ml of 10% disodium EDTA solution for 24 hr. The EDTA was discarded and the procedure repeated twice with fresh EDTA. Each demineralized femur half was blotted with tis-

TABLE I. EFFECT OF LEVELS OF DIETARY GLUTAMIC ACID (GLU) ON GROWTH RATE, FEED CONSUMPTION, FEED EFFICIENCY, AND PLASMA AMINO ACID CONCENTRATIONS

	Treatment	
Amino acid	5% Glu	12.5% Glu
7-day weight gain (g)[a]	42.3	51.4
Feed consumption (g)[a]	64.7	71.3
Gain/feed[a]	0.66	0.72
Plasma amino acids[b]		
Aspartic acid	19.3	236.7
Glutamic acid	160.8	1193.0
Proline	248.0	548.5
Alanine	981.1	2675.1
Glycine	1002.8	1502.7
Hydroxyproline	103.8	221.1
Indispensable amino acids[c]	2312.8	2444.1

[a] Average values for 28 chicks/treatment. Treatment differences were not statistically significant ($P > 0.10$).
[b] Average values of pooled plasma samples from 7 chicks/treatment expressed as nanomoles per milliliter.
[c] Includes His, Thr, Arg, Val, Met, Cys, Ile, Leu, Phe, Trp, and Lys.

sue paper, transversely sectioned, placed in another counting vial, and digested with 3 ml of Soluene-350. Following complete digestion, 18 ml of Dimilume-30 was added and the samples were counted as described for calvaria. The counts obtained for each femur half from both femurs were added together and reported as total counts for both femurs from each bird.

Statistical significance was determined by analysis of variance (6).

Results and Discussion. Although the differences were not statistically significant, 7-day weight gains and feed efficiency were poorer and feed consumption was lower for chicks fed 5% than for those fed 12.5% glutamic acid (Table I). Since feed efficiency was poorer, the consumption of all nutrients, with the exception of glutamic acid, per unit of weight gain was greater in chicks fed the lower level of glutamic acid.

Increasing the dietary level of glutamic acid from 5 to 12.5% elevated the free dispensable amino acid levels in the plasma (aspartic acid, glutamic acid, alanine, proline, and glycine) with the greatest response noted with aspartic and glutamic acids (Table I). These results are in general agreement with a previous report where dietary levels of 5 and 9.7% glutamic acid were compared (1).

Plasma hydroxyproline concentrations were also higher in chicks fed the 12.5% glutamic acid diet than in those fed the 5% glutamic acid diet (221 and 104 nmoles/ml, respectively). Since plasma hydroxyproline originates from preformed collagen, the results suggest a greater turnover or remodeling of collagen in chicks fed the higher level of glutamic acid.

TABLE II. EFFECT OF LEVELS OF DIETARY NONSPECIFIC NITROGEN ON THE INCORPORATION OF [^{14}C]GLUTAMIC ACID (GLU) INTO CALVARIA PROTEIN *in Vitro*[a]

Fraction and treatment			
EDTA extract		EDTA residue	
5% Glu	12.5% Glu	5% Glu	12.5% Glu
5.50	10.68*	4.06	11.38**

[a] Expressed as nanomoles incorporated per 100 mg of calvaria. Correction was made for specific activity based on the plasma glutamic acid concentration presented in Table I.

* 12.5% Glu significantly different from 5% Glu ($P < 0.06$).

** 12.5% Glu significantly different from 5% Glu ($P < 0.01$).

TABLE III. EFFECT OF LEVELS OF DIETARY NONSPECIFIC NITROGEN ON THE INCORPORATION OF [^{14}C]PROLINE INTO FEMURS *in Vivo*[a]

Treatment	
5% Glu	12.5% Glu
120.95	303.33**

[a] Expressed as nanomoles incorporated into both femurs. Corrected for plasma proline concentrations presented in Table I. It was assumed that 7.3% of a 2-week-old chick's body weight is plasma and the specific gravity of plasma is 1.02 (7).

** Significantly different from 5% Glu ($P < 0.01$).

Incorporation of [^{14}C]glutamic acid into the phenol extract of the EDTA-soluble fraction of calvaria was greater ($P < 0.06$) in chicks fed 12.5% glutamic acid than in chicks fed 5% glutamic acid (Table II). Similarly, higher incorporation ($P < 0.01$) of glutamic acid into the residue following EDTA extraction of calvaria was noted for chicks fed the elevated level of glutamic acid (Table II).

In vivo studies with [^{14}C]proline incorporation into femurs followed the same trend observed with *in vitro* incorporation of [^{14}C]-glutamic acid into calvaria protein (Table III). Intraperitoneally injected [^{14}C]proline incorporation into the EDTA-extracted femurs of chicks fed 12.5% glutamic acid was much greater ($P < 0.01$) than that incorporated into femurs of chicks fed 5% glutamic acid.

In agreement with previous studies (1), the incidence of moderate to severe leg problems in the present investigation was 36% for chicks fed 5% glutamic acid as compared with 0% in those fed 12.5% glutamate. The 36% incidence of leg problems in chicks fed 5% glutamic acid is considerably lower than that reported earlier (1, 2); however, the present evaluation was made at 7 days as compared with 14 days. It has been noted that the incidence and severity increases between 7 and 14 days.

From these results, it can be concluded that bone protein synthesis (and possible turnover or remodeling) is dramatically influenced by the level of nonspecific nitrogen in the diet. The connection between this observation and the myriad of leg anomalies observed in chicks fed low levels of total nitrogen is not known. It would appear that the results are not merely due to a reduction in total protein synthesis since additional glutamic acid corrected the leg anomalies in chicks fed toxic levels of seven D-amino acids without influencing growth rate (2). Furthermore, with a diet low in nonspecific nitrogen similar to that used in the present study, the addition of nitrogen as diammonium citrate overcame the leg problems without improving growth rate.

1. de Moraes GHK, Rogler JC, Featherston WR. Effects of nonspecific nitrogen deficiency on growth rate and leg problems in chicks. Poult Sci **63**:344–353, 1984.
2. de Moraes GHK, Rogler JC, Featherston WR. Effects on D-amino acids on growth rate and kidney D-amino acid oxidase in chicks. Poult Sci **66**:98–102, 1987.
3. Price PA, Otsuka AS, Poser JW, Kristoponis J, Ramon N. Characterization of a γ-carboxyglutamic acid-containing protein from bone. Proc Natl Acad Sci USA **73**:1447–1451, 1976.
4. Landt M, Butler LG. 5′ nucleotide phosphodiesterase: Isolation of covalently bound 5′ adenosine monophosphate, an intermediate in the catalytic mechanism. Biochemistry **17**:4130–4135, 1978.
5. Waters Division of Millipore Corp. Pico-tag Amino Acid Analysis System-Operator's Manual (Part No. 88140), Revision 3. Milford, MA, Millipore Corp., Waters Chromatography Division, 1986.
6. Steel RGD, Torrie JH. Principles and Procedures of Statistics. New York, McGraw–Hill, 1980.
7. Sturkie PD. Avian Physiology. New York, Cornell Univ. Press, 1965.

Received November 2, 1987. P.S.E.B.M. 1988, Vol. 188.
Accepted February 22, 1988.