THE SPECIFICITY OF THE ^{3}H-PROLINE INCORPORATION TEST AS A MEASURE OF BONE MATRIX FORMATION.

Sandy C. Marks, Jr.
Dental Research Dept., Naval Medical Research Inst.,
National Naval Medical Center, Bethesda, Md. 20014

Received March 24, 1969

Summary - The fate of ^{3}H in mice given ^{3}H-proline has been examined by amino acid and radiometric analyses of calvarial bone hydrolysates. The proline and hydroxyproline fractions contained 80% of the isotope, and ^{3}H-hydroxyproline represented 25% of the total bone radioactivity. These findings indicate that bone matrix formation may be evaluated accurately in the calvaria by measuring the amount of isotope per mg of bone.

A popular method for measuring bone formation is quantitative assay for labeled hydroxyproline after administration of its precursor, labeled proline (Flanagan and Nichols, 1962). This reliable index of bone collagen formation is based upon the facts that hydroxyproline in animal tissue is found almost exclusively in collagen and that it is formed by hydroxylation of peptide-bound proline (Udenfriend, 1966). However, the method requires isolation of hydroxyproline by hydrolysis and chromatography (Firschein, 1967). Walker (1966) has proposed a simplified procedure, the ^{3}H-proline incorporation test, which produces consistent results. Animals are sacrificed six hours after the intraperitoneal administration of the isotope, after which the calvaria is removed and cleaned of all adherent soft tissue (figure 1). Weighed aliquots of pulverized bone are then emulsified at room temperature in 2.0 ml Hyamine 10-X

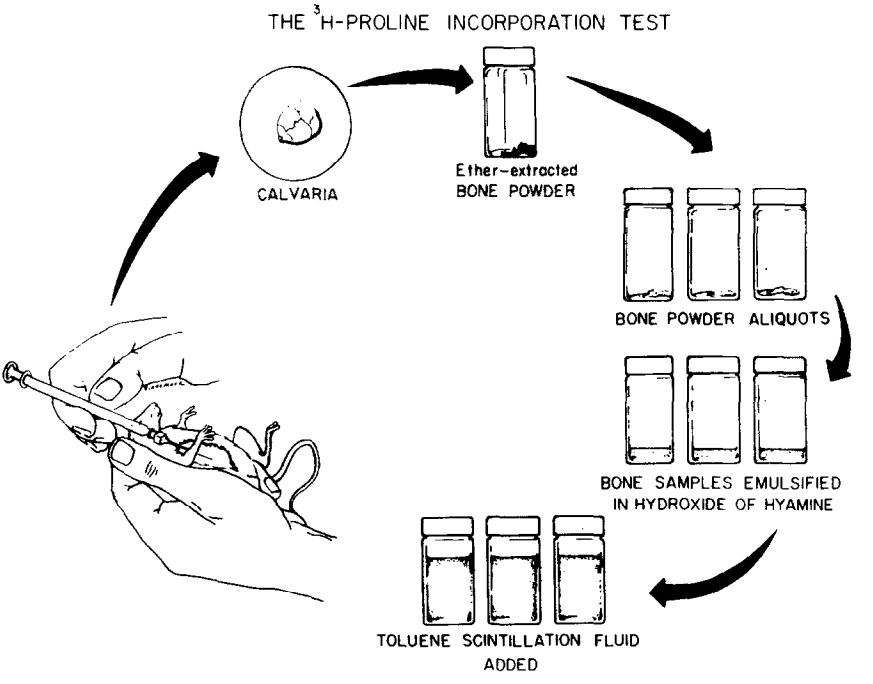

Figure 1 - The ^{3}H-proline incorporation test.

(hydroxide), Rohm and Haas trademark for p-(diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium hydroxide, prior to counting. The ^{3}H-proline incorporation test measures the total radioactivity of calvarial bone, expressed as the net counts per min. per mg.

The advantage of this method is simplified sample preparation. However, the ^{3}H-proline incorporation test assumes that the isotope is present in bone as part of proline or hydroxyproline and that labeled hydroxyproline, the most direct measure of new bone collagen formation, is a constant percentage of the total radioactivity. In the present investigation these assumptions have been validated on the basis of combined radiometric and amino acid analyses.

Materials and Methods - Normal (NIH/NMRI) and osteopetrotic (Dickie, 1967) mice were given L-proline-3,4-^{3}H or (UL) L-proline-^{14}C (of radiochemical purity greater than 99.5%) from New England Nuclear Corp. at a dose of 4.0 microcuries per gram body weight. The mice were killed by decapitation 6 hours after the administration of the isotope. Calvariae free of soft tissue were dried and pulverized with fine scissors. Five-milligram aliquots, hydrolyzed at 110° for 22 hours in 1.0 ml 6N HCl, were evaporated to dryness and taken up in a suitable volume of buffer. Amino acid analysis was performed by ion-exchange chromatography (Beckman Model 120B), and a stream-divider was used to collect fractions of the column effluent for radioactivity measurements. The liquid scintillation system used had an efficiency for tritium of 17%.

Results - A typical chromatogram of mouse calvarial bone hydrolysate is shown in the upper trace of figure 2 and below this the location of the two radioactive peaks found in the analysis for neutral and acidic amino acids. Radioactivity was restricted to the hydroxyproline and proline fractions. The results of several types of experiments are shown in table 1. The ratio of proline to hydroxyproline in all samples, when either ^{3}H or ^{14}C were used, remained approximately 2.2:1. This suggests that the relative amount of hydroxylated proline was constant and that there was no detectable tritium exchange to other amino acids from carbons 3 and 4 of the pyrrolidine ring. There was close correspondence between the ratio of proline to hydroxyproline whether determined by amino acid analysis or radio-

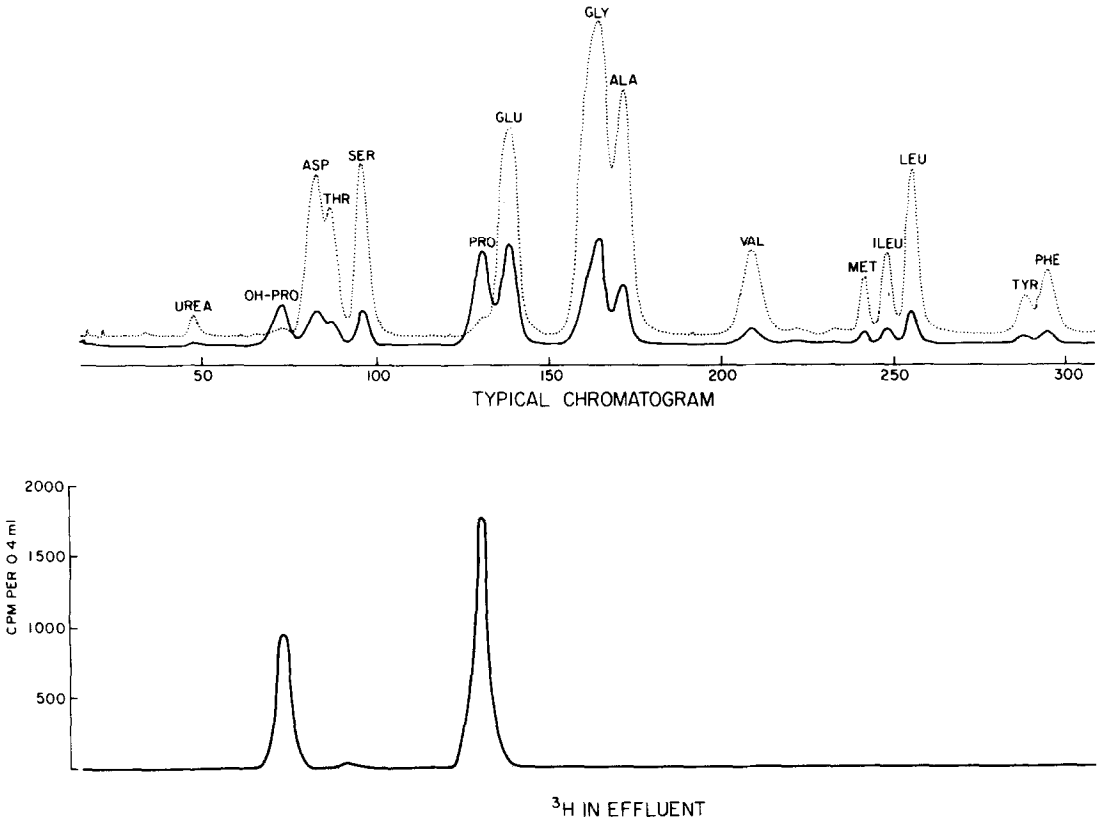

Figure 2 - Chromatogram of acidic and neutral amino acids in the calvarial bone hydrolysate from an 18-day-old albino mouse given ^{3}H-proline. Shown below is the location and amount of radioactivity measured in fractions of the column effluent.

activity measurement. Radioactive hydroxyproline represented approximately 25% of the total radioactivity in calvarial bone from normal mice and mice with an accelerated rate of bone matrix synthesis (osteosclerotic and experimentally osteopetrotic). Finally, approximately 80% of the isotope present in each sample was located in the proline and hydroxyproline peaks, a yield consistent with the work of others (Flanagan and Nichols, 1962). Analysis for basic amino acids revealed that most of the remaining radioactivity was located in the peak represented by

TABLE 1

THE FATE OF RADIOACTIVITY IN CALVARIAL BONES FROM MICE GIVEN THE ^{3}H-PROLINE INCORPORATION TEST.

	RATIO OF PROLINE TO HYDROXYPROLINE AS DETERMINED BY:		PROPORTION OF TOTAL RADIOACTIVITY REPRESENTED BY:	
	SCINTILLATION COUNTING	AMINO ACID ANALYSIS	HYDROXYPROLINE	PROLINE + HYDROXYPROLINE
^{3}H-PROLINE UPTAKE IN ALBINO MICE:	2.28	2.21	24.1%	79.0%
	2.26	2.21	24.0%	77.7%
^{14}C-PROLINE UPTAKE IN ALBINO MICE:	2.24	2.35	24.6%	78.4%
	2.16	2.24	24.1%	76.0%
^{3}H-PROLINE UPTAKE IN:				
OSTEOSCLEROTIC MOUSE	2.35	2.41	22.2%	72.6%
NORMAL LITTERMATE	2.16	2.24	22.7%	72.4%
EXPERIMENTALLY OSTEOPETROTIC MOUSE	2.34	2.30	24.6%	82.1%
NORMAL LITTERMATE	2.26	2.34	25.5%	83.2%

TABLE 2

COMPARISON OF TOTAL BONE RADIOACTIVITY AND LABELED HYDROXYPROLINE IN NORMAL AND OSTEOPETROTIC MICE GIVEN ^{3}H-PROLINE

	TOTAL DPM/MG CALVARIAL BONE	DPM OH-PROLINE/MG CALVARIAL BONE
OSTEOSCLEROTIC MOUSE	7153	1588
NORMAL LITTERMATE	4547	1023
EXPERIMENTALLY OSTEOPETROTIC MOUSE	6423	1580
NORMAL LITTERMATE	4429	1129

arginine, a known product (Stetten, 1955) of proline metabolism via ornithine. Thus, the great majority of counts in each sample were present in proline and hydroxyproline with the amount of the latter imino acid being 25% of the total radioisotope in bone.

A comparison of the results of the ^{3}H-proline incorporation test and a radiometric analysis of labeled hydroxyproline in the same animal is presented in table 2. The ratio of total DPM/mg in osteosclerotic mouse calvarial bone to that of its normal littermate was 1.57:1 and the ratio of the hydroxyproline radioactivities was 1.54:1. Similar values for the experimentally osteopetrotic mouse and its littermate were 1.45:1 and 1.40:1 respectively.

Discussion - The results reported here indicate that the ^{3}H-proline incorporation test as outlined by Walker (1966) is a valid expression of ^{3}H-hydroxyproline and thus of new bone matrix formation.

The 2.2:1 proportion of proline to hydroxyproline observed in this study is greater than the reported 1:1 ratio found in chick bone collagen (Miller and Martin, 1968). It should be emphasized that the analyses were performed on pulverized mouse calvarial bone and not pure bone collagen, and the discrepancy may be due to this fact and the species difference.

Two years elapsed from the time of administration of ^{3}H-proline and the analysis for hydroxyproline in the mice of osteosclerotic stock (table 1). The low values found in these two mice for the percentages of total radioactivity represented

by hydroxyproline and proline + hydroxyproline may be due to tritium exchange over this period. Nevertheless, the results of the ^{3}H-proline incorporation test agree with the analyses for radioactive hydroxyproline performed two years later (table 2).

Acknowledgements - This investigation was supported by U. S. Naval Medical Research Institute project no. MR005.19-6057B. The technical assistance of Mr. V. J. Berzinskas is gratefully acknowledged. The opinions and assertions contained herein are the ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. Experiments were conducted according to the principles set forth in the "Guide for Laboratory Animal Facilities and Care" prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences--National Research Council.

References

Dickie, M. M., Mouse News Letter #36, p.39, 1967.
Firschein, H. E., Arch. Biochem. Biophys. 119:119-123, 1967.
Flanagan, B. and G. Nichols, Jr., J. Biol. Chem. 237:3686-3692, 1962.
Miller, E. J. and G. R. Martin, Clin. Orthopaed. #59:195-232, 1968.
Stetten, M. R., in A Symposium on Amino Acid Metabolism, ed. W. D. McElroy and B. Glass (Baltimore:Johns Hopkins, 1955) p.277.
Udenfriend, S., Science 152:1335-1340, 1966.
Walker, D. G., Endocrinology 79:836-843, 1966.