lated from the 90-day old tissue of the male rat there is a similar reduction. Specific activity of hydroxylysine in collagen of the 300-day old tissue of the male is 26% that of 17-day old tissue, while in the 300-day old tissue of the female, 55% of the 17-day tissue value was observed. This would seem to indicate that the female connective tissue has as active an hydroxylating mechanism at 300 days as found at 50 days of tissue age. It would appear that the tissue of the male has a reduction in rate of hydroxylation with tissue age. Yet it has been demonstrated that no increase in collagen content occurs after 21 days of tissue growth in the female rat, while the male tissue continues to accumulate collagen to 300 days of tissue age(6). This suggests that either rate of conversion of lysine to hydroxylysine is not an index of collagen formation or that the female tissue contains some substance which enhances breakdown of collagen at the same rate as its synthesis.

Summary. 1. C^{14}-labeled lysine administered intraperitoneally into the rat is incorporated within hours into the saline-soluble and -insoluble non-collagenous protein and collagen of biopsy-connective tissue. Radioactivity is confined to lysine in all fractions of connective tissue except for a portion which is converted to hydroxylysine in the collagen. 2. Following appearance of radioactivity in all fractions of connective tissue, decay occurs rapidly in saline-insoluble non-collagenous protein and saline-soluble protein and slowly in the collagen fraction. Thirty days after injection of labeled lysine approximately 10% of maximum activity remained in the saline protein fractions while 30% remained in collagen. 3. Incorporation of labeled lysine into the non-collagenous protein of connective tissue is not influenced by tissue age while its incorporation into the collagen appears to decrease with tissue age. 4. Specific activity of labeled hydroxylysine from a 300-day old tissue of a female rat was approximately twice that of a comparably aged tissue from a male rat.

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In vitro Incorporation and Hydroxylation of Lysine-2-C^{14} In Rat Biopsy-Connective Tissue.* (24096)

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Labeled lysine administered orally or intraperitoneally to the rat appears in the collagen fraction of connective tissue as lysine and hydroxylysine(1,2). The purpose of the present study was to determine the *in vitro* incorporation and hydroxylation of lysine-2-C^{14} in con-

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nective tissue obtained by the sponge biopsy technic(3) and to compare results with those obtained from *in vivo* studies. The study of hydroxylation of lysine *in vitro* provides an adaptable technic for study of biosynthesis of collagen.

Methods. Three polyvinyl sponges (Ivalon‡) were implanted subcutaneously along lower dorsum of 6-month-old Sprague-Dawley rats by a previously described technic(3). At different intervals of tissue age, the sponge-connective tissue was removed. *In vitro* studies. The sponges were removed from a male and a female rat 18 days following implantation, from a female rat after 24 days and from 2 male rats after 34 days. The sponge-connective tissue was immediately placed into ice-cold Krebs-Henseleit solution (4), which had been gassed 30 minutes with 95% O_2:5% CO_2. The tissue was kept chilled during preparation for incubation. The capsule was removed and sponge-connective tissue was sliced as thin as possible with razor blade. Approximately 500 mg of tissue was placed in 25 ml Erlenmeyer flask containing 5 ml of Krebs-Henseleit solution. One ml of neutralized solution of lysine-2-C^{14} in physiologic saline (0.255 mg dl-lysine-2-C^{14} monohydrochloride or 1 μc) was added to each flask. After the flasks were gassed with 95% O_2:5% CO_2 for one minute and capped with a rubber stopper, they were incubated, with constant agitation, in 37°C water bath. At 2-hour intervals, the flasks were removed and gassed. To study rate of lysine incorporation, flasks containing slices of 34-day-old tissue were removed in duplicate from water bath after 0, 1, 2, 4, 6, 8, and 12 hours incubation and immediately placed in freezer at –28°C. Other tissues were incubated 12 hours. To determine the effect of a complete amino acid mixture upon conversion of lysine to hydroxylysine, tissue culture medium #199§ without bicarbonate(5) was used as incubation medium instead of Krebs-Henseleit solution. Slices of two 24-day-old tissues from a female rat were equally divided among 6 incubation flasks, 3 containing 5 ml of Krebs-Henseleit solution and 3 containing 5 ml of #199 medium. One ml of neutralized lysine-2-C^{14} was added to each flask, and the flasks incubated 12 hours. *In vivo* studies. Sponge-connective tissue was obtained from 2 male rats 90 days after sponge implantation, after 17 days of connective tissue growth from a male and a female rat and after 50 days growth in female rat. All animals received a single intraperitoneal injection of one ml of neutralized dl-lysine-2-C^{14} monohydrochloride (9.45 mg or 0.024 mc) in physiological saline. The rats were sacrificed and tissues removed 8 hours following injection. *Tissue analysis.* Tissues were removed from incubation mixture and blotted to remove excess lysine-2-C^{14}. The blotted tissue slices and tissues removed in the *in vivo* experiments were prepared for analysis by homogenizing in physiologic saline containing 0.25% lysine in a VirTis "45" macro homogenizer‖ for 5 minutes with rheostat reading at 50. The homogenates were centrifuged at 15,000 x g for one hour. The saline-soluble protein of supernatant was precipitated with 4 volumes of 10% trichloroacetic acid (TCA). The saline-insoluble residue was washed once with physiologic saline and again centrifuged at 15,000 x g. The supernatant was discarded and the residue separated into collagen and non-collagenous protein by the hot 0.3 M TCA extraction technic of Fitch *et al.*(6). The precipitated protein of the saline-soluble fraction, collagen and non-collagenous protein were hydrolyzed with 6 N HCl at 110°C for 16 hours in sealed pyrex tubes. Nitrogen was determined by Conway microdiffusion method(7) and converted to protein value by the 6.25 factor. Hydrolysates were evaporated on water bath to remove excess hydrochloric acid, neutralized and made up to volume. One ml of neutralized hydrolysate was evaporated to dryness on aluminum planchet for determination of radioactivity of the protein fraction. The remainder of the hydrolysate was evaporated to dryness and redissolved in citrate buffer pH 5.0 and placed on a 15 cm column of Dowex 50-x8 according to the method of Moore and

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TABLE I. Rate of Incorporation of Lysine-2-C^{14} in Rat Biopsy-Connective Tissue.

Sex	Age of tissue (days)	Time of incubation (hr)	Specific activity (cpm/mg of protein): Saline-soluble protein	Saline-insoluble residue: Collagen	Saline-insoluble residue: Non-collagenous protein
♂	34	0	54	26	5
		1	808*	42	431
		2		73	923
		4	1,827*	76	—
		6		176	2,000
		8	4,650	216	2,875
		12	3,020	426	4,705
♂	34	0	71	24	44
		1	528*	68	396
		2		75	678
		4	1,892*	149	1,910
		6		143	1,970
		8	3,336	370	3,735
		12	3,202	683	3,123
♀	24	12	4,465	421	4,203
♂	18	12	—	460	4,703
♀	18	12	—	409	3,376

* Combined samples. —, not done.

Stein for basic amino acid chromatography (8). Aliquots from each fraction of the eluate were dried on aluminum planchets for measurement of radioactivity. Specific activity of lysine and hydroxylysine was calculated on the basis of ninhydrin reaction (9) of aliquots of each eluate fraction.

Results. Rate of incorporation of lysine-2-C^{14} into biopsy-connective tissue is shown in Table I. Specific activities of saline-soluble protein and of non-collagenous protein increased with length of incubation and plateaued in most tissues after 8 hours of incubation. Specific activity of collagen appeared to increase to 12 hours of incubation. Incorporation rate of younger tissue, *i.e.*, 18 days and 24 days, seemed to approximate that of 34-day-old tissue.

Chromatographic separation of the basic amino acids indicated that lysine-2-C^{14} was incorporated into lysine of the saline-soluble protein, of collagen and of non-collagenous protein of biopsy-connective tissue and was converted to labeled hydroxylysine in the collagen fraction. These findings for the *in vitro* system are similar in this respect to the results of the *in vivo* study (2).

Comparison of *in vitro* and *in vivo* study of incorporation and hydroxylation of lysine-2-C^{14} is made in Table II. Specific activities of the 3 protein fractions of tissues of the *in vitro* study were greater than those of tissues of the *in vivo* study. Specific activity of lysine in collagen isolated from tissues studied *in vitro* was approximately 10 times greater than that of collagen from older tissue (50 and 90 days of age) and 5 times that of young tissue (17 days of age) studied *in vivo*. Conversion of labeled lysine to hydroxylysine occurred in incubated tissue slices although a relatively smaller amount of lysine incorporated into collagen was hydroxylated when compared with injected rats. In *in vivo* studies, there

TABLE II. Comparison of *In Vitro* Study with *In Vivo* Study of Incorporation and Hydroxylation of Lysine-2-C^{14} in Biopsy-Connective Tissue.

	Sex & No. of tissues	Age of tissue (days)	Specific activity (cpm/mg protein or amino acid): Saline-soluble protein (Protein)	Saline-insoluble protein: Collagen (Protein)	Collagen (Lysine)	Collagen (Hydroxylysine)	Non-collagenous protein (Protein)
In vitro (12-hr incubation)	♂ 1	34	3,020	426	4,722	1,679	4,705
	♂ 1	34	3,202	683	4,601	1,148	3,123
	♀ 1	24	4,465	421	3,827	1,779	4,203
In vivo (8 hr after inj.)	♂ 2	90	230	48	335	310	249
	♀ 1	50	216	62	487	397	266
	♂ 1	17	151	64	858	939	233
	♂ 1	17	139	74	784	804	166

TABLE III. Incorporation of Lysine-2-C^{14} in Krebs-Henseleit Solution and in Tissue Culture Medium #199.

	Specific activity (cpm/mg protein)		
		Saline-insoluble residue	
	Saline-soluble protein	Collagen	Non-collagenous protein
Krebs-Henseleit solution	4,465	425 ± 197*	4,203 ± 699
#199 medium	1,570	127 ± 34	1,661 ± 165

* Mean and stand. dev. of 3 tissues.

was a 1:1 ratio of specific activity of labeled lysine to that of tagged hydroxylysine in collagen while in collagen of the *in vitro* study a ratio of 4:1 existed.

Comparison of incorporation and hydroxylation of lysine-2-C^{14} in tissue slices incubated in Krebs-Henseleit solution and in tissue culture medium #199 is made in Table III. Specific activities of protein fractions of tissues incubated in Krebs-Henseleit medium are significantly higher than those in the #199 medium.

Discussion. Labeled lysine can be demonstrated to be incorporated *in vitro* into tissue proteins of preformed connective tissue. Labeled lysine not only becomes incorporated into the collagen fraction but also is hydroxylated and appears as labeled hydroxylysine. *In vitro* formation of hydroxylysine indicates a biochemical process beyond incorporation or attachment of an amino acid, such as lysine, as an end group of a protein molecule and suggests the formation of at least a peptide which becomes an integral portion of collagen since hydroxylysine is found only in collagen.

Specific activities of the saline-soluble and non-collagenous protein increased with incubation time up to 8 hours while the lower specific activity of collagen increased to 12 hours. Incubation was not continued beyond 12 hours because of the possibility of growth of bacterial contaminants. The fact that there is an increase in radioactivity with time in the protein fractions further suggests that incorporation of the labeled lysine involves a metabolic process and is not a mere attachment through an epsilon linkage to a protein molecule.

The much greater specific activity of lysine in tissues studied *in vitro* than that of *in vivo* studies may be related in part to the larger concentration of labeled amino acid presented to tissue in the incubation flask. If the specific activity or weight of the injected labeled lysine were related to the approximate protein content of a 200 g rat, about one-eighth as much radioactive lysine was available to tissue of the intact rat as was placed in the incubation flask. In addition, labeled lysine administered to the intact animal would be further diluted by inactive lysine of the body.

Comparison of incorporation of labeled lysine into connective tissue incubated in Krebs-Henseleit with that incubated in a synthetic amino acid mixture (#199), indicates a significantly greater incorporation in Krebs-Henseleit medium. Since the synthetic amino acid mixture contained 7 mg % of unlabeled lysine (*i.e.*, 0.35 mg/5 ml of #199 medium), this finding is not surprising because dilution of the labeled amino acid and competition, such as occurred in *in vivo* experiments, by all proteins for labeled and non-labeled lysine would no doubt occur. The uniform depression of lysine incorporation into the saline-soluble and non-collagenous proteins as well as into collagen suggests a lack of augmentation of collagen synthesis by a commonly used and supposedly more ideal medium for tissue culture work, *i.e.*, tissue culture medium #199.

Specific activity of collagen from tissue of the *in vivo* experiments was a greater percentage of total specific activity of the 3 protein fractions than was that of tissues of the *in vitro* study. Specific activity of collagen of the *in vivo* experiments was approximately 20 to 50% that found either in saline-soluble or in non-collagenous protein, while collagen from *in vitro* studies was approximately 10% that found in either of the other 2 protein fractions. Specific activity of lysine in collagen of the *in vitro* study was 3 to 4 times higher than that of the hydroxylysine, while specific activity of lysine of collagen of the *in vivo* study was about equal to that of the hydroxylysine. These findings suggest that while collagen of *in vivo* studies had a lower specific activity than that of *in vitro* experiments, the former system converts lysine to hydroxylysine more effectively and thus pro-

vides an index of extent of collagen formation.

Summary. 1. Lysine-2-C^{14} becomes incorporated and hydroxylated in connective tissue incubated in Krebs-Henseleit medium for 12 hours. 2. Specific activity of collagenous and non-collagenous protein in the tissue studied *in vitro* was greater than that in tissue studied *in vivo*. 3. Hydroxylation of labeled lysine appears to occur more effectively in connective tissue of rats given labeled lysine intraperitoneally than in the connective tissue incubated with lysine-2-C^{14}.

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Elevated Vitamin Levels in Cerebrospinal Fluid in Multiple Sclerosis.* (24097)

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Very few studies have been reported on vitamin content of CSF (cerebrospinal fluid) in health and in disease. Since vitamins supply the prosthetic groups of enzymes, observations of their absence, or inability of absorption or transmission to the proper tissues may be forged into tools to elucidate specific metabolic dysfunctions; vice versa their pathological accumulation may be correlated with hyperfunction(1). Cyanocobalamin and folic acid (PGA) are known to provide the enzymatic apparatus for important steps in the synthesis of nucleic acids. Disturbances in distribution of these vitamins may interfere with the transfer of methyl groups and upset the equilibrium between ethanolamine and choline, or between the respective phosphatides cephalin and lecithin(2). These chemical changes in turn may give rise to demyelination, a histological picture of obscure, but undoubtedly enzymatic etiology(3).

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Methods. The quantities of these vitamins in body fluids, tissues and organs are of such minute order of magnitude that conventional methods of chemical analysis cannot be used, but one has to resort to microbiological assay (4). The analysis of PGA offers particular intricacies, since PGA occurs conjugated to variable extent with peptides of variable molecular weight, requiring enzymatic deconjugation previous to microbioassay; the enzymatic material which must be introduced is itself a source of folic acid which considerably complicates the analysis. We have described (5) a microbioassay, using a thermophilic bacillus which grows rapidly on a simple, purely synthetic medium and which responds equally well to various conjugates as to free folic acid, thus obviating previous enzymatic deconjugation. In the case of Vit. B_{12} preference has hitherto been given to the flagellate *Euglena gracilis* over various strains of *Lactobacillus* used for this purpose, because of its higher sensitivity and specificity(6). However, even *Euglena* shows a wider spectrum than appears desirable, as it responds to certain congeners of Vit. B_{12}, which are ineffective in man and