of incorporated tritium labelled thymidine is distributed to the daughter cell at mitosis(1) one can expect that by administering tritium labelled thymidine at 6 hour intervals, virtually all cells dividing during the period between booster injection of antigen and sacrifice would be exposed to, and incorporate labelled thymidine. The value of 92% stated as the labelling index of the plasma cell series here is probably conservative and may actually approach 100%. This low value might result from the initiation and completion of DNA synthesis in some cells between exposures to isotope or from technical limitations in the sensitization of the radiographic emulsion by the label in some cells. These factors, alone or in combination, would tend to lower the observed incidence of labelling. The results presented are in general agreement with those of other investigators employing similar technics(8,10,11) who have implicated a rapidly proliferating population of immature lymphoblasts and plasmablasts as the chief source of plasma cells arising after antigenic stimulation. As a corollary, the large percentage of labelled antibody containing cells found in these experiments indicate that most plasma cells do not arise by direct, non-mitotic differentiation from small lymphocytes. The present studies could not offer quantitative data concerning the relative mitotic activity of the various stages leading to development of the mature plasma cell. However, by the use of colchicine and by short *in vitro* incubation of cells with thymidine it has been shown that functionally differentiated antibody producing cells can divide, indicating that mitotic activity within the family of antibody producing cells is not an exclusive property of the immature or precursor forms.

Summary. A technical approach for the study of the kinetics of antibody producing cells is possible by combination of autoradiography and the fluorescent antibody technic. Results show that: 1. Almost all antibody containing cells present by day 4 of a secondary response are newly formed cells arising from mitotic division of a precursor sometime after antigenic stimulation; 2. Most plasma cells do not arise by direct, non mitotic differentiation from lymphocytes; and 3. Functionally differentiated antibody containing cells can divide.

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Connective Tissue VI: Synthesis of Collagen by Rat Uterine Slices.* (27084)

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One of us(1) reported *in vitro* synthesis of collagen by sponge biopsy connective tissue. A year later, Green and Lowther(2) ob-

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served the *in vitro* synthesis of collagen by carrageenin granulomata. So far, none of the normal tissues of the body has been shown to synthesize collagen *in vitro*. Recently we demonstrated that, of a series of rat tissues tested *in vivo*, the uterus showed the highest capacity for collagen synthesis (3). The present study is concerned with the synthesis of collagen by uterine slices.

Methods. Slices of uterus, to a total of 0.2-0.5 g, from Wistar strain rats were incubated in 5 ml Krebs-Henseleit medium(4). To this, in each instance, was added 1 ml saline containing amounts of uniformly labelled C^{14}-lysine‡ varying in total radioactivity as noted in Table I. Incubation proceeded at a temperature of 37°C; pH varying from 7.35 to 7.45; and an atmosphere of O_2, 95% and CO_2, 5% in a Dubnoff incubator.§ After incubation, for varying periods of time as noted, the slices were removed from the medium, blotted with filter paper to remove excess C^{14}-lysine and homogenized with 10 ml saline containing 0.25% non-radioactive lysine in an ice chilled VirTis "45"‖ homogenizer for 5 minutes. The homogenized mixture was centrifuged at 27,000 × g at 15°C for 30 minutes. The supernatant was combined with the incubation medium and studies of these combined solutions will be reported elsewhere. Collagen was extracted from the residue twice with 0.3 M trichloroacetic acid (TCA), at 90°C by Fitch's method(5). The final residue was nonscleroprotein. This report deals only with the collagen fraction.

The hydrolysis of collagen, estimation of protein and determination of radioactivity were accomplished by methods as previously reported(3). An aliquot of collagen hydrolysate, containing 10-15 mg of collagen amino acids was subjected to basic amino acid chromatography according to the method of Moore and Stein(6). Each chromatographed fraction was checked for amino acid content and radioactivity by previously reported methods(1,3,7,8). In early experiments, the

‡ As purchased from Nuclear-Chicago, Inc. this contained 3 μc of activity per μmole of lysine.

§ American Instrument Co., Silver Spring, Md.

‖ Clay-Adams, New York City.

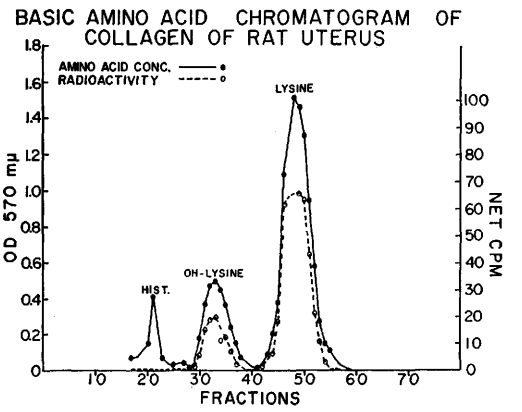

FIG. 1. Amount of amino acid was determined by the Moore and Stein method(7). Optical density was read at 570 mμ. This figure has been constructed from the net counts/min., and does not take into account our counting efficiency, which is 23.44%.

hydroxylysine eluates were desalted and rechromatographed. The radioactivity was proved to be in the hydroxylysine region. Our counting efficiency is 23.44% and our counting error is 2.5%. The amount of radioactive hydroxylysine present was accepted as a measure of collagen synthesis.

Results. Fig. 1 is the basic amino acid chromatogram of collagen of rat uterus. It demonstrates the synthesis of collagen by conversion of C^{14}-lysine to C^{14}-hydroxylysine.

In Table I is shown the influence of varied amounts of C^{14}-lysine on the radioactivity of the collagen, hydroxylysine and lysine isolated. As the amount of radioactivity was increased from 0.625 to 1.250 μc, specific activities (cpm/mg protein or amino acid) of the collagen and hydroxylysine formed were approximately doubled. The specific activity of lysine was not quite doubled. Further addition of C^{14}-lysine caused no further increase in specific activities.

The influence of duration of incubation on synthesis of collagen by rat uterine slices is shown in Table II. At zero hour, no synthesis of collagen had occurred, as indicated by the absence of radioactivity in hydroxylysine; the radioactivity appearing in the collagen at this time was due to adsorption of C^{14}-lysine. As measured by formation of hydroxylysine, definite synthesis of collagen was observed after 3 hours of incubation. When

TABLE I. Influence of Amount of C^{14}-Lysine on Collagen Synthesis by Rat Uterine Slices.

Radioactivity added* (μc)	Specific activity (cpm/mg) Collagen	Hydroxylysine	Lysine†
	Exp. 1‡		
.625	657	2735	10887
1.250	1327	6753	17329
1.875	1263	6148	15815
	Exp. 2‡		
.625	1655	7146	25431
1.875	4198	12755	43131

* As C^{14}-lysine. The preparation employed contained 3 μc of radioactivity per μmole of lysine. The indicated amount was always dissolved in 1 ml of saline for addition to incubation medium.

† These figures represent total radioactive lysine present, which includes amount of lysine incorporated into the collagen molecule plus that adsorbed by the collagen.

‡ Donor rats were one yr old. Slices were incubated in Krebs-Henseleit solution at 37°C, pH 7.35 to 7.45 for 12 hr in 1st experiment and 6 hr in 2nd experiment.

the period of incubation was increased to 6 hours, the specific activity of the hydroxylysine fraction was nearly doubled. A further increase in time of incubation to 12 hours was not associated with further increment in the specific activity of collagen, hydroxylysine or lysine. When a 24-hour period of incubation was employed, the newly formed collagen was less in amount than at 12 hours. This may have been the result of destructive bacterial action.

TABLE II. Influence of Period of Incubation upon Synthesis of Collagen by Rat Uterine Slices.*

Time (hr)	Collagen	Hydroxylysine	Lysine†
0	358	0	2427
3	939	5354	10768
3	909	3605	10269
6	1182	7961	11429
6	1199	7018	10094
12	1399	7884	15094
12	1310	5282	6310
24	2227	4279	16020
24	2154	—	—

* One-yr-old rats were used as donors. Incubation medium contained 1.875 μc of C^{14}-lysine. For this experiment slices from uteri of 9 rats were employed and so pooled as to represent an even distribution of tissue from each animal in each incubation flask.

† These figures represent total radioactive lysine present, which includes amount of lysine incorporated into the collagen molecule plus that adsorbed by the collagen.

From the data presented in Table III, it is evident that the capacity for synthesizing collagen in uterine slices is an inverse function of age of the donor animal.

Discussion. From the present studies it is clear that collagen synthesis can be achieved *in vitro* through the use of rat uterine slices. Depending upon the conditions of the individual experiment, the specific activity of lysine in the newly formed collagen is either equal to or higher than that of hydroxylysine. When the specific activity of lysine is higher this is probably in part due to adsorption phenomena. Such adsorbed C^{14}-lysine can not be completely removed by dialyzing

TABLE III. Synthesis of Collagen by Uterine Slices from Rats of Several Ages.

Age of rat	Specific activity (cpm/mg) Collagen	Hydroxylysine	Lysine†	% of incorp. in hydroxylysine*
		Exp. 1‡		
6 wk§	4578	32334	31924	.0640
1 yr	1399	7884	15094	.0252
	1310	5282	6310	.0192
2 "	316	346	5000	.0256
	516	687	6988	.0299
		Exp. 2‡		
4 wk§	8938	11318	36711	.0623
1 yr	2150	1485	15064	.0145
	2462			
2 "	926	363	5222	.0085
	947			

* % incorporation in hydroxylysine =

$$\frac{\text{Specific activity of hydroxylysine} \times \text{mg of hydroxylysine} \times 100}{\text{cpm of } C^{14}\text{-lysine added to incubation medium}}$$

† These figures represent total radioactive lysine present, which includes amount of lysine incorporated into the collagen molecule plus that adsorbed by the collagen.

‡ In Exp. 1 duration of incubation was 12 hr; 1.875 μc of C^{14}-lysine were added to incubation medium. In Exp. 2 duration of incubation was 6 hr; 0.625 μc of C^{14}-lysine was added to incubation medium.

§ Combined sample from 4-5 rats.

agents, water or M NaCl. However, washing with 5% TCA removes all radioactivity, thus simultaneously removing the newly synthesized collagen. Recently, working with skin Schultz-Haudt and Eeg-Larsen have demonstrated the presence of "neutral heteropolysaccharide-protein complexes" containing

appreciable amounts of hydroxyproline which are removed from animal skin by 5% TCA (9). They believe such complexes play an important role in the laying down of collagenous tissue and that the hydroxyproline present actually represents soluble collagen.

A word should be said about the plateauing of radioactivity in the several fractions (collagen, hydroxylysine and lysine) with increments in the amount of C^{14}-lysine added. While it is not easy to calculate the amount of lysine and its hydroxy-derivative present in the protein of the uterine slices of each flask we have reason to believe that measured as lysine somewhere between 1900 and 4800 μg are present per flask. Whenever we add 1 μc of radioactive lysine we are simultaneously adding 50 μg of lysine in its free and "chemically pure" state. This would represent roughly between 1 and 2% of all the lysine present. Under the conditions of our experiment, we believe this represents an appreciable quantity, capable of influencing the endpoints of enzymic activity. Since no further increase in total radioactivity as hydroxylysine appears when the radioactivity is increased from 1.25 to 1.875 by addition of C^{14}-lysine we believe that the enzymic systems present have been maximally utilized. This experiment has been repeatedly performed as indicated by examples shown in Table I. The results are always the same.

From the data here presented it will be observed that the younger the uterine donor the better the incorporation of lysine into hydroxylysine. At 6 weeks, the specific activity of the parent amino acid is either equal to or 3 times that of its hydroxy-derivative, whereas in the 2-year-old donor the specific activity of the precursor reaches 10 times that of the derivative. This clearly demonstrates the lowered capacity of aged tissues for forming collagen.

We believe the uterine slice technic can probably be standardized as a method of bioassay in which each pool of uterine slices can be so divided and incubated as to serve for control and test purposes simultaneously.

Summary. Under standardized conditions the formation of soluble collagen has been followed in uterine slices. Maximal incorporation of radioactivity under the conditions stated has been achieved by 1.250 μc of C^{14}-lysine for an incubation period of 6 hours in Krebs-Henseleit solution at 37°C, with a pH range between 7.35 and 7.45 and an atmosphere of 95% oxygen and 5% CO_2.

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