into the milk(5). In subsequent clinical studies of nursing mothers, it was reported that therapeutic dosages of bishydroxycoumarin could be administered without danger of hemorrhage or effect on prothrombin time in the nursing infants(8,9). The present experiments on warfarin sodium in rats are consistent with the above results, and suggest that therapeutic dosages of warfarin sodium could probably be administered to nursing mothers without deleterious effect on the infants.

Summary. When a therapeutic dosage of the anticoagulant warfarin (Coumadin) sodium was fed to lactating rats, there was no effect on whole blood clotting time of the nurslings, or on their subsequent growth and reproduction. When toxic or lethal levels of warfarin sodium were fed to the mothers, clotting time was prolonged in the nurslings. This demonstrated that the anticoagulant effect could be transmitted through the milk when mothers received toxic dosages. However, at all dosages the anticoagulant effect was much less in nurslings than in mothers.

1. Shapiro, S., *Surg. Clin. N. Am.,* 1956, v36, 469.
2. McCarthy, A. M., *West. J. Surg., Obst. and Gynec.,* 1959, v67, 64.
3. Quick, A. J., *Am. J. Clin. Path.,* 1940, v10, 222.
4. Connell, W. F., Mayer, G. A., *Canad. Med. Assn. J.,* 1959, v80, 785.
5. Quick, A. J., *J. Biol. Chem.,* 1946, v164, 371.
6. Council on Drugs, A.M.A., *New and Nonofficial Drugs, 1960,* J. B. Lippincott Co., Philadelphia, 1960, p507.
7. Field, J. B., *Am. J. Physiol.,* 1945, v143, 238.
8. Barker, N. W., Hines, E. A., Jr., Kvale, W. F., Allen, E. V., *Am. J. Med.,* 1947, v3, 634.
9. Brambel, C. E., Hunter, R. E., *Am. J. Obst. and Gynec.,* 1950, v59, 1153.

Received July 5, 1960. P.S.E.B.M., 1960, v105.

Site of Formation of Thyroglobulin in Mouse Thyroid as Shown by Radioautography with Leucine-H^3.* (26001)

N. J. NADLER, C. P. LEBLOND AND J. CARNEIRO†
Department of Anatomy, McGill University, Montreal, Canada

Locating sites of protein synthesis in the thyroid gland has been attempted after injection of S^{35}-labelled methionine(1,2), but may now be done better with tritium (H^3)-labelled amino-acids, since the soft beta-rays of tritium allow a more precise radioautographic localization than those of S^{35}. When mice were injected with methionine-, glycine- or leucine-H^3, the location of the H^3 label in the thyroid was found to be the same with the 3 amino-acids but reactions were more intense with leucine-H^3 than with the other 2. This last result was not unexpected since leucine has been listed(3) as the most abundant amino-acid in thyroglobulin, the characteristic iodinated protein found in the thyroid colloid. The next step was to investigate quantitatively the thyroid gland of those mice which had been treated with leucine-H^3. This was done in the present investigation.

Methods. Ten adult male mice weighing 26-32 g received a single injection of 5 μc of DL-leucine-4, 5-H^3 (150 mc/mM) per gram body weight and were sacrificed in pairs at ½, 4 and 35 hours, 7 and 45 days later. Thyroid glands were fixed in Bouin, stained with hematoxylin-eosin and radioautographed(4).

In one section of each thyroid gland, the largest and smallest diameters of the colloid of every follicle were measured with a micrometer; and photographic grains were counted within a 10.4 x 10.4 μ area over the center of the colloid. Grains were then counted within 2 x 10.4 μ areas selected over 2 diametrically opposed portions of the epithelium of each follicle. Finally, grains were also counted in 10.4 x 10.4 μ areas located outside the thyroid

* Supported by a grant of Nat. Research Council of Canada. The assistance of Mr. R. G. Fletcher is acknowledged.

† Rockefeller Fellow, now at: Dept. de Histol. e Embriol., Fac. de Med., Univ. de Sao Paulo, Brasil.

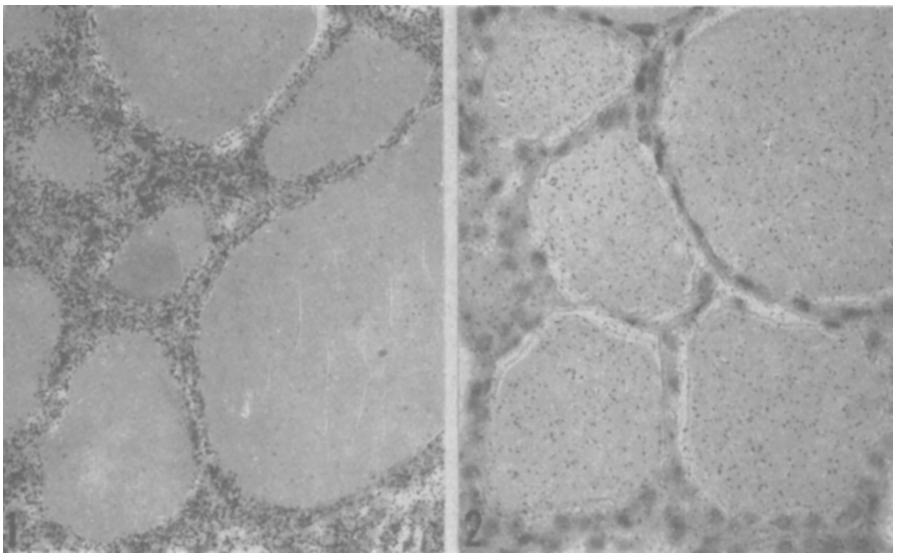

FIG. 1. Coated radioautograph of a hematoxylin-eosin stained section of thyroid gland from an adult mouse, sacrificed ½ hr after inj. of leucine-H^3. Abundant silver grains overlie epithelium but not colloid of thyroid follicles. (The rare grains found over the colloid are attributable to the background fog of the emulsion.)

FIG. 2. As in Fig. 1, except the animal was sacrificed 35 hr after inj. of leucine-H^3. Grains over colloid are much more abundant than at early time interval (Fig. 1).

gland, as an estimate of the background fog of the emulsion to be deducted from the counts over colloid and epithelium.

The grain counts expressed per unit area were grouped for follicle sections in which mean colloid diameter was 20 μ or less, 21-40. 41-60, 61-80, 81-100, 101-120 or greater than 120 μ. Since the diameters of the follicle sections did not necessarily represent the true diameters of the follicles from which they were derived, the grain counts were corrected as done previously(5)‡ and referred to 6 follicle classes (Tables I and II).

TABLE I. True Concentration of Leucine Label in *Epithelial Cells* of Thyroid Follicles, from Radioautographic Grain Counts.

Colloid diam., μ	Grain counts per unit area at various times after admin. of radio-leucine				
	½ hr	4 hr	35 hr	7 days	45 days
40	69.2	23.8	24.4	—	2.7
60	31.6	21.7	23.1	8.4	4.7
80	36.7	26.9	22.9	7.1	5.0
100	44.2	29.3	24.1	5.8	4.0
120	30.3	—	30.8	10.2	4.4
140	50.4	25.0	20.1	10.0	4.4

TABLE II. True Concentration of Leucine Label in *Colloid* of Thyroid Follicles, from Radioautographic Grain Counts.

Colloid diam., μ	Grain counts per unit area at various times after admin. of radio-leucine				
	½ hr	4 hr	35 hr	7 days	45 days
40	.7	12.1	28.0	—	1.6
60	.6	4.8	15.3	9.8	7.9
80	1.1	5.2	10.3	5.4	6.9
100	1.4	4.0	3.1	5.3	5.3
120	—	5.2	4.8	15.1	5.8
140	1.5	2.7	5.2	4.3	3.7

Results. At a half hour after injection, the leucine label was present throughout the epithelium of every thyroid follicle (Fig. 1), while at 35 hours and later it appeared in the colloid (Fig. 2).

The corrected or "true" concentration of label in the cells at any given time interval (Table I) was not significantly influenced by diameter of the follicles. Concentration was maximal at a half hour after injection and decreased with time, rapidly between ½ and 4 hours, and slowly thereafter.

The true concentration of the label in the colloid (Table II) was insignificant at the half hour interval, then increased with time in all follicle classes. A maximum was reached at 35 hours in the 3 smaller follicle classes.

‡ Raw data and methods used in calculations reported in this article will be supplied on request.

By 45 days, radioactivity had decreased in all follicles.

Discussion. Following administration of radioactive amino-acids, the radioactive label appears in newly-formed proteins(6), site of synthesis of which can be demonstrated by radioautography(1). Accordingly, the radioautographic reaction observed over the epithelium of all follicles soon after a single injection of leucine-H^3 (Fig. 1) demonstrated that all epithelial cells of the thyroid gland were synthesizing protein.

The radioautographic grain count over the epithelium decreased after ½ hour, indicating loss of the newly-formed protein (Table I). Analysis revealed that this loss occurred rapidly in an early phase (turnover time, 7.5 hours) and more slowly thereafter (turnover time, 19.7 days), suggesting production in the epithelium of at least 2 types of new protein.

During the early phase, the decrease in concentration of labelled protein in the epithelium coincided with an increase in the colloid (Table II). Calculation of radioactivity in the entire epithelium and colloid at ½ and 35 hours revealed that epithelial content decreased by 135 and colloid content increased by 117 arbitrary units of radioactivity between the 2 time intervals. The rather close correlation implied that the loss of labelled protein from the epithelium could be accounted for by simultaneous appearance of labelled protein in the colloid. The small relative difference between the figures for epithelium and colloid, if at all significant, may mean that not all the labelled substance turned over by the epithelium in the first phase was deposited into the colloid, and/or some of the labelled substance deposited in the colloid had already been itself turned over in this interval.

By virtue of the similarity between the properties of isolated thyroid colloid(7) and purified thyroglobulin(8), it is believed that the main constituent of thyroid colloid is thyroglobulin. And, since leucine has been reported to be the most abundant amino-acid in thyroglobulin(3), the leucine-containing protein synthesized by the cells, then rapidly released to the colloid, must be the precursor of thyroglobulin. Now, the organic binding of iodine in the thyroid takes place in the colloid(9,10); hence, the formation of typical thyroglobulin (containing the iodinated amino-acids from which thyroid hormone is derived) must occur after the precursor reaches the colloid.

The much less rapid turnover of the label which took place in the epithelium during the later phase (turnover time of 19.7 days) had no similar counterpart in the colloid. Rather, the slow loss was similar to that reported for cells of many organs and was likewise attributed to the internal turnover of cellular proteins(1). It was estimated that about one-half of the labelled protein made in the epithelial cells was thus used up in intracellular metabolism, while the other half consisted of the thyroglobulin precursor to be deposited into the colloid.

Analysis of the turnover of leucine label in the colloid of each follicle size class suggested presence of only one type of labelled substance, that is, newly-formed thyroglobulin (whatever its degree of iodination). While cells of all follicle classes seemed to synthesize precursor at about the same rate (Table I), a greater rate of concentration of newly-formed thyroglobulin was observed in the colloid of small follicles as opposed to large follicles (Table II), due to the fact that there is a greater volume of epithelium per unit volume of colloid in smaller than in larger follicles (5).

The eventual decrease with time in colloid content of labelled protein in all follicles (Table II) may be attributed to the proteolytic breakdown of thyroglobulin (as a result of which thyroid hormone is liberated(11).

Summary. Ten adult male mice were injected with tritium-labelled leucine and sacrificed in pairs at various time intervals thereafter. Thyroid gland sections were radioautographed by the coating technic and photographic grains counted over epithelium and colloid. The leucine label is incorporated into protein by every cell of every thyroid follicle at 30 minutes after injection, but its concentration in the cells decreases thereafter. Concentration of labelled protein in the colloid was insignificant at ½ hour, increased and reached a maximum at 35 hours or soon

thereafter, and subsequently diminished. The appearance of the leucine label in colloid of all follicles examined between ½ hour and 35 hours could be accounted for by simultaneous loss of label from the epithelial cells. Since leucine is the most prominent amino-acid in thyroglobulin(3), the results are interpreted to represent the synthesis of this protein in the epithelium and its secretion into the colloid.

1. Leblond, C. P., Everett, N. B., Simmons, B., *Am. J. Anat.*, 1957, v101, 225.

2. Karpishka, Irene. Sites of protein synthesis as shown by radioautographic distribution of methionine labelled with C^{14} or S^{35} in mice and rats. M.Sc. thesis, Dept. of Anatomy, McGill Univ., 1958.

3. Haurowitz, F., *Chemistry and Biology of Proteins*, Acad. Press Inc., New York, 1950, 32.

4. Messier, B., Leblond, C. P., Proc. Soc. Exp. Biol. and Med., 1957, v96, 7.

5. Nadler, N. J., Leblond, C. P., Bogoroch, R., *Endocrinology*, 1954, v54, 154.

6. Tarver, H., Peptide and Protein Synthesis. Protein Turnover, in H. Neurath and K. Bailey, *The Proteins*. Acad. Press, Inc., New York, 1199-1292.

7. Leblond, C. P., Glegg, R. E., Eidinger, D., *J. Histochem. Cytochem.*, 1957, v5, 445.

8. Ujejski, L., Glegg, R. E., *Can. J. Biochem. Physiol.*, 1955, v33, 199.

9. Nadler, N. J., Leblond, C. P., *Brookhaven Symp. in Biol., The Thyroid*, 1955, v7, 40.

10. Wollman, S. H., Wodinsky, I., *Endocrinology*, 1955, v56, 9.

11. Gross, J., Leblond, C. P., *ibid.*, 1951, v48, 714.

Received July 5, 1960. P.S.E.B.M., 1960, v105.

6-Beta-Hydroxy-Cortisol: High Levels in Human Urine in Pregnancy and Toxemia.* (26002)

Andrew G. Frantz,† Fred H. Katz‡ and Joseph W. Jailer§

Departments of Medicine and Obstetrics and Gynecology, Columbia University College of Physicians and Surgeons, and Presbyterian Hospital, New York City

6-beta-hydroxy-cortisol (6β-OH-F) has been shown by Burstein to exist as a major metabolite of cortisol in guinea pig urine(1). He also detected its presence in minute amounts in normal human urine and in larger amounts in urine excreted during late pregnancy and after cortisol administration to normal males(2,3). Colle and Ulstrom have recently found significant quantities of 6β-OH-F in urine of the human newborn(4). The high degree of polarity conferred by the extra hydroxyl group renders 6β-OH-F difficult to extract from aqueous media with many conventional organic solvents and greatly hinders its chromatographic separation in systems designed for less polar corticosteroids. To overcome these difficulties, new methods of extraction and chromatography have been devised and the following experiments were performed using such technics.

Methods and materials. Fresh unhydrolyzed urine is extracted with ethyl acetate after prior addition to the urine of 20% by weight of sodium sulfate; this salt is also added in similar concentrations to the alkali and aqueous washes which follow. Recovery experiments and determination of partition coefficients validated the efficiency of extraction of 6β-OH-F by this method. Chromatography is carried out in 2 successive highly polar solvent systems, the first using chloroform:ethyl acetate:methanol:water; and the second, benzene:*tert.*-butanol:water. After elution from the second paper, quantitation is accomplished by the Porter-Silber reaction. Hydrolysis of the urine with β-glucuronidase before extraction did not increase the yield in several experiments. Identity and homogeneity of the 6β-OH-F recovered were confirmed by a number of technics in which an authentic sample was compared; these include chromatographic mobilities and color reac-

* Aided by Grant A-2208 (c) from Nat. Inst. Health, U.S.P.H.S.

† Trainee, Inst. of Arthritis and Metabolic Dis., N.I.H. Now on active duty, U. S. Navy.

‡ Fellow of National Foundation, 1959-60.

§ Deceased.