only ones through which *feed-back* control of immunoglobulin synthesis can operate. Therefore, under physiological conditions, the combined effect on regulation of antibody synthesis of all these structural features, might still be sufficiently specific without involving the antibody combining sites.

Summary. Regulation of immunoglobulin synthesis by *feed-back* inhibition was investigated so as to test whether the specificity of *feed-back* inhibition depends on recognition of allotypic specificities. This inhibition was observed in rabbits which were pretreated by neonatal injections with antiserum directed against their paternal allotypic specificity. As a result of this pretreatment, synthesis of immunoglobulin of paternal type was reduced (suppressed) for 3-4 months. After this time, some of the pretreated rabbits were injected with normal rabbit serum, containing immunoglobulin of the suppressed type. This resulted in an inhibition of the synthesis of this allotypic immunoglobulin lasting from 50 to 150 days. It was concluded that *feed-back* inhibition of immunoglobulin synthesis may operate through recognition of allotypic specificities and possibly through recognition of other structural features of immunoglobulins as well.

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1. Pfeiffer, R., Dtsch. Med. Wschr., 1901, v27, 867, 891.
2. Uhr, J. W., Baumann, J. B., J. Exp. Med., 1961, v113, 935.
3. Rowley, D. A., Fitch, F. W., ibid., 1964, v120, 987.
4. Dubiski, S., Dubiski, A., Skalba, D., Kelus, A., Immunology, 1961, v4, 236.
5. Dray, S., Dubiski, S., Kelus, A., Lennox, E. S., Oudin, J., Nature, 1962, v195, 785.
6. Gurvich, A. E., in Immunological Methods, J. F. Ackroyd, Ed., Blackwell, Oxford, 1964, p113.
7. Oudin, J., Meth. Med. Res., 1952, v5, 335.
8. Cinader, B., Dubiski, S., Wardlaw, A. C., J. Exp. Med., 1964, v120, 897.
9. Brambell, F. W. R., Hemmings, W. A., Henderson, M., Parry, H. J., Proc. Roy Soc., 1949, vB136, 131.
10. Dray, S., Nature, 1962, v195, 677.
11. Mage, R., Dray, S., J. Immunol., 1965, v95, 525.
12. Gell, P. G. H., Kelus, A., Nature, 1962, v195, 44.
13. Rieder, R. F., Oudin, J., J. Exp. Med., 1963, v118, 627.
14. Oudin, J., Michel, M., C. R. Acad. Sci., Paris, 1963, v257, 805.
15. Gell, P. G. H., Kelus, A. S., Nature, 1965, v201, 687.
16. Lark, C. A., Eisen, H. N., Dray, S., J. Immunol., 1965, v95, 404.
17. Allen, J. C., Kunkel, H. G., Kabat, E. A., J. Exp. Med., 1964, v119, 453.
18. Kunkel, H. G., Mannik, M., Williams, R. C., Science, 1964, v140, 1218.

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Further Studies on the Incubation of Tyrosine-UL-C^{14} with Beef Thyroid Tissue Slices.* (31071)

MORRIS DILLARD,† DOROTHY R. HOLLINGSWORTH, AND PHILIP K. BONDY
Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.

Phenylalanine is considered to be one of the essential amino acids, although tyrosine, its para-hydroxylated derivative, can substitute for it. The major site at which phenylalanine is converted to tyrosine is the liver. Subsequent utilization follows several pathways, one of which is believed to be the synthesis by the thyroid of thyroxine and its precursor analogs. The order of magnitude of

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this incorporation was recently implied when only 1 part in 50,000 of intraperitoneally injected universally labeled-C^{14} tyrosine was incorporated into thyroglobulin(1). This low value raises the question of the capacity of the thyroid gland to utilize this amino acid. These experiments were designed to study the incorporation of tyrosine-UL-C^{14} by thyroid tissue slices into thyroxine and its related compounds.

Procedure. Beef thyroid glands were obtained fresh from a local abattoir and chilled during 20 minutes of transport. The adventitia was removed and tissue slices made with a Stadie Riggs microtome. Randomized tissue slices weighing 0.5 g were added to each of three 100-ml flasks, each containing 50 ml of Ringer bicarbonate buffer, pH 7.4(2) and 100 microcuries of UL-C^{14}-tyrosine,‡ previously added to ethanol to the individual flasks and dried under air.

The experimental design was as follows: Flask 1 contained only the material listed; Flask II contained 11 mg of propylthiouracil, and Flask III received 10 μg of chromatographically pure thyronine. A control flask without tissue slices, containing UL-C^{14}-tyrosine, was also incubated. Incubation at 37°C in a Dubnoff metabolic shaker was continued for 2 hours under an atmosphere of 5% CO_2 and 95% oxygen. The reaction was stopped by addition of 2 M sodium hydroxide until the pH was 8.4. Two hundred fifty mg of pancreatin obtained from Mann Research Laboratories was added to each flask and the top was layered with toluene. The flasks were covered and incubated 48 hours at 37°C. The digests were filtered through fine sintered glass filters and the residue washed with N-butanol (chemically pure) until no further radioactivity was extracted.

The extracted materials were carried to dryness under air. Each of the individual samples was subjected to counter current distribution, paper chromatography, and finally thin layer chromatography.

Counter current procedure. The apparatus employed was a one hundred-tube model made by the E.C. Apparatus Co. The solvent system was prepared by equilibrating 1500 ml of 3 N ammonium hydroxide with 1500 ml of N-butanol for 24 hours. Ten ml aliquots of each phase were added to each tube with the exception of the first. To this tube was added the sample which was transferred with 10 ml of each of the phases. The rate of transfer was determined by the appearance of a clean interface. Ninety-nine transfers were carried out. The tubes were emptied into test tubes and 3 ml aliquots from the upper phase were taken for spectrophotometric analysis at 280 mμ in a Beckman Model DU spectrophotometer. Radioactivity was counted in a Packard tricarb scintillation counter on a 0.05 ml aliquot of the upper phase pipetted directly in the counting vial and dried under air. The distribution of the absorbency values was plotted and compared to values previously obtained for monoiodotyrosine (MIT), diiodotyrosine (DIT), diiodothyronine (T_2), triiodothyronine (T_3), thyroxine (T_4), tyrosine and thyronine, when separated from a chemical mixture containing equal quantities of each. The radioactivity was plotted against the absorbing values of its respective sample.

Paper chromatography. Following counter current distribution, the fractions were pooled into 4 groups as follows: Group I—tubes 3 to 20; Group II—tubes 21 to 55; Group III—tubes 56 to 80; Group IV—81 to 100. Tubes one and two had very little radioactivity and contained the residual protein following digestion. They were excluded from analysis. The pooled fractions were dried under air and spotted on Whatman No. 1 paper for chromatography prepared in 23-inch lengths and 6-inch widths that were cut into one-inch fingers to avoid cross contamination. Chemical controls of tyrosine, MIT, DIT, thyronine, T_2, T_3, and T_4 were applied as a mixture on strips between the experimental samples. The solvent system employed was 50 ml tertiary butanol and 50 ml 2 N NH_4OH. The paper was placed into a conventional glass chromatography tank and mobile phase(3) added. Migration was allowed for 12 hours at room temperature. The paper was removed, dried in air, and the radioactivity localized by scanning in a Packard model 7200 strip scanner.

‡ The radioactive tyrosine was obtained from New England Nuclear Corp. with a specific activity of 0.10 millicurie per 0.0503 mg. Its purity was demonstrated by the paper and thin layer chromatographic systems used in other analyses in this study.

Chemical controls were located with ninhydrin reagent.

Thin layer chromatography. Following localization of radioactivity from the paper chromatography, the strips were sectioned into the individual peak areas, eluted with alkaline N-butanol, dried under air, and spotted on glass plates prepared for chromatography(4). The supporting medium used was cellulose and the solvent system in which the plates were developed was an ascending system of tert-butanol (326 ml):2 N ammonium hydroxide (74 ml):chloroform (60 ml). Between each sample a chemical control mixture identical to that for paper chromatography was spotted. The plate was equilibrated in a saturated tank for 2 hours. Mobile phase was carefully poured into the bottom and allowed to ascend for 10 hours. The plate was removed and dried in air. The areas corresponding to the samples were covered with aluminum foil and the remainder of the plate sprayed with ninhydrin. The sample areas, defined as one-inch strips from three-eighths of an inch below the origin to the top of the plate, were then exposed. This width was subsequently divided crosswise into one-eighth-inch lengths. Each area was scraped from the plate, put into counting vials, scintillator added and counted in the scintillation counter. The resulting counts were graphed and the location of the radioactivity was compared with the location of the ninhydrin spots of the intermixed chemical controls. All three experimental extracts were treated identically.

Results. Following the extraction of the digested incubation mixture with N-butanol, the recovery of radioactivity was greater than 99.9% for each of the individual flasks. Following counter current separation the radioactivity was found principally in 3 areas, when pooled referred to as Groups I, II, and IV. No difference in the results between the 3 experimental designs was apparent. Each column was evaluated as to ultraviolet absorption (represented by the heavy line) and radioactivity (represented by the light line) (Fig. 1). Three major radioactive peaks are demonstrated: Group I—tubes 3 to 20; Group II—tubes 21 to 55; Group IV—81 to 100. Tubes 56 to 80 referred to as Group III had no radioactivity but was analyzed further by chromatography systems. Two major absorption peaks are likewise demonstrated. The absorption distribution was the same between the 3 experiments, but correlation between the radioactivity and its own absorption plot was not exact. The first of the 2 absorption peaks appeared slightly before radioactive Group II and the second peak just beyond Group IV.

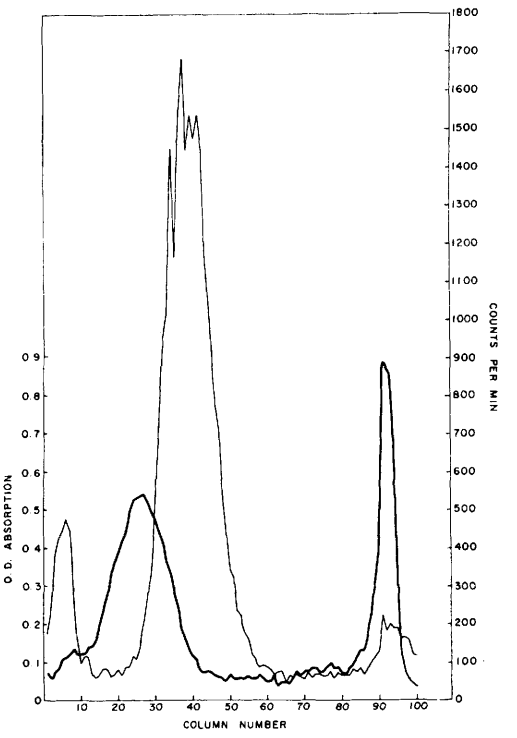

FIG. 1. Ultraviolet absorption (heavy line) and radioactivity (light line) of counter current separation of hydrolysate from Exp. 1. Three major radioactive peaks are demonstrated: Group I tubes 3-20; Group II tubes 21-55; Group IV tubes 81-100. Tubes 56-80, referred to as Group III, had no radioactivity but were analyzed further by chromatography.

Group II from the counter current distribution of thyroid hydrolysate was applied to paper and chromatographed in an alkaline tertiary butanol system. The radioactivity was distributed in many peaks (Fig. 2). The pooled extracts from Group I, when treated similarly, failed to move from the origin, and those from Group IV traveled with the solvent front. No radioactivity was present in Group III. Therefore, after paper chromatography no radioactivity was localized near the thyronine, iodotyrosine or iodothyronine areas in

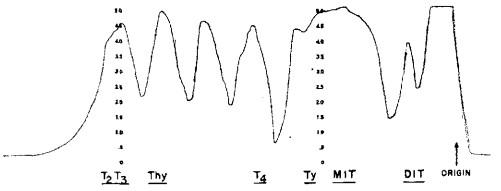

FIG. 2. Scan of radioactivity of Group II compounds after paper chromatography from Exp. 1. Location of chemical standards is indicated below the trace of the scan. These read, from left to right: di-iodo- and tri-iodothyronine, thyronine, thyroxine, tyrosine, mono-iodotyrosine and di-iodotyrosine.

Groups I, III or IV. This distribution was found in all 3 experimental designs.

When the areas of Group II following paper chromatography were eluted and rechromatographed by thin layer, no radioactivity was detected more than two-eighths of an inch from the origin. Although the chemical controls separated well, there were no areas of radioactivity corresponding to the iodotyrosine or thyronine standards.

Discussion. Under the conditions of these experiments there is no evidence for the formation of thyroxine or other iodinated derivates from tyrosine. Recovery of radioactivity excludes the possibility of loss through technique. The compound was isolated unchanged from the control flask, containing only labeled tyrosine and no tissue slices, but subjected to pancreatin digestion. Destruction of this group of compounds by proteolysis has been suggested(5) but the amount of thyroxine loss represented less than thirty per cent. Thus, while breakdown during hydrolysis may play some role in the quantitative interpretation of results it does not explain a complete absence of the expected iodotyrosines or iodothyronines.

The iodinated thyronines were not expected in the propylthiouracil experiment but failure to isolate the thyronine skeleton suggests failure of coupling by whatever mechanism it may occur. With the addition of thyronine to the incubation mixture, one interpretation could be that the chemical material was incorporated before the radioactive counterpart could be formed. But this explanation is doubtful since no one has demonstrated the incorporation of thyronine into thyroxine, and under our experimental procedure no thyronine analogs were formed from the radioactive substrate with or without addition of chemical thyronine.

Instead of forming thyroxine and its analogs, the tyrosine substrate molecule was converted into a number of unknown compounds. By counter current distribution there were 3 radioactive areas, but Group II was the only one whose components separated in the chromatography systems used. This single group had at least 7 distinct areas in the chromatogram, none of which corresponded to the thyroid compounds or their precursors. Since this experiment was designed to study tyrosine and its incorporation into thyroid analogs, only those systems which would give isolation and separation of these particular compounds were used. It is reasonable to consider that if different chromatography systems had been employed, Group I and Group IV would have separated into a like series of compounds; however, there is no reason to suspect that iodotyrosines or thyronines would have been demonstrated.

Such active metabolism by the thyroid of the tyrosine molecule into compounds other than thyroxine and its precursor analogs was totally unexpected. Approximately 90 to 95% could readily be accounted for in these conversions. Although the nature of these breakdown products in thyroid gland is unknown, in the liver, deamination and various oxidation-reduction attacks on the side chain ultimately give compounds common to Krebs aerobic energy cycle. From this pathway a large number of other compounds may be formed and this is probably what has been observed in these experiments.

From the point of view of the data of these *in vitro* studies, certain questions may be asked of the *in vivo* studies(1). Following intraperitoneal injection of UL-C^{14}-tyrosine, only one part in fifty thousand of the radioactivity was incorporated into the gland. Attempts to identify the amino acid carrying this radioactivity were not performed. To assume that it was tyrosine or an analog would be unwise. In view of the fact that most of the tyrosine had to pass through the liver in Maloof's experiment, a large amount of catabolic products would be expected, and it is reasonable to postulate that a very small amount of tyrosine remained for incorporation

into thyroxine and its precursor analogs if, indeed, any such incorporation occurred.

Summary. 1. Beef thyroid tissue slices were incubated in the presence of 100 microcuries UL-C^{14}-tyrosine with 3 variations: a) no additions, b) propylthiouracil addition, c) nonradioactive thyronine. 2. Following digestion, analysis proceeded through counter current, paper and thin layer chromatography. The results for each of the experimental designs were the same. 3. Of the 3 major areas of radioactivity seen after counter current, only one could be further subdivided by the systems usually used for separation of iodotyrosines, and iodothyronines, and this yielded at least 7 peaks in the systems used. 4. No evidence was found for *in vitro* formation of thyroxine or any of its precursors using tyrosine as the labeled substrate.

Since this work was completed, Nunez, Mauchamp, Macchia and Roche (Biochim. Biophys. Acta, 1965, v107, 247) have also reported that they were unable to demonstrate incorporation of tritiated tyrosine into the iodotyrosines of thyroglobulin formed by sheep thyroid slices. Since the tritium label of tyrosine is stable only on the alanine sidechain, this observation is specific only for the alanine chain on the tyrosyl residue attached to the thyroglobulin skeleton. The use of UL^{14}C-tyrosine in our experiments also affords information about the ether-linked distal iodophenol.

1. Maloof, F., Sato, G., Soodak, M., Medicine, 1964, v43, 375.

2. Umbreit, W. W., Burris, R. H., Stauffer, J. F., Manometric Techniques, Fourth Edition, 1964.

3. Dillard, M., Hollingsworth, D. R., Bondy, P. K., Proc. Soc. Exp. Biol. and Med., 1965, v119, 905.

4. Hollingsworth, D. R., Dillard, M., Bondy, P. K., J. Lab. & Clin. Med., 1963, v62, 346.

5. Rosenberg, L. L., LaRoche, G., Endocrinol., 1964, v75, 776.

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Collagen and Hexosamine Changes in Subcutaneous Granuloma Irradiated Locally with a Co^{60} Source.* (31072)

MARCEL E. NIMNI,† CHARLES LYONS AND LUCIEN A. BAVETTA‡

Department of Biochemistry and Nutrition, School of Dentistry, University of Southern California, Los Angeles

We have recently reported that γ-radiation from sealed sources of Co^{60} embedded inside developing granulomas was able to decrease the amount of soluble collagen at this site (1). Although this reflected a drop in collagen synthesis it did not seem to affect the amount of insoluble collagen present, presumably because the level of radioactivity used was relatively low (55 and 180 μc of Co^{60}).

In the present series of experiments we intended to pursue this study using a more radioactive source of Co^{60}, and in addition to investigate changes in the skin just above the subcutaneous granulomas as well as remote from the area of implantation.

Methods. Radiation source. Stainless steel capsules, 6 mm long and 1.5 mm in diameter, were loaded with 1 mC of Co^{60}. Fig. 1 shows diagrammatically the radioactive capsule positioned in the center of a plastic sponge. The isodose radiation curves were calculated on the basis of theoretical attenuation and experimental data compiled by Fletcher *et al* (2). Empty capsules were used as controls.§

Experimental procedure. Adult Holtzman male rats weighing 360 to 400 g were implanted subcutaneously with polyvinyl sponges (Ivalon) weighing 100 mg ± 2 mg. The sponges were sterilized by autoclaving, allowed to dry and at this time a small hole

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§ Capsules supplied by U.S. Nuclear Corp., Burbank, Calif.