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Rabbit Thyroid Metabolism in Tissue and Organ Culture.* (32159)

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Mosier(1) recently reported the inability to detect labeled iodothyronine in the tissue or media of surviving rabbit thyroid tissue incubated in media containing iodide I-131. He was able to detect labeled iodothyronine in surviving *rat* thyroid tissue however, under the same *in vitro* conditions. Previous reports of the metabolism of C^{14} tyrosine in media with surviving rabbit and beef thyroid tissue and tritiated tyrosine in media with sheep thyroid slices have been conflicting(1-5).

The results of the present studies fail to support the concept that a species difference exists in the metabolism of iodide I-131 by thyroid tissue. In addition, we have been unable to detect incorporation of C^{14}-labeled phenylalanine or tyrosine into iodotyrosines or iodothyronines by thyroid tissue grown in organ culture in the presence or absence of TSH.

Methods. Adult New Zealand female rabbits were fed Purina Rabbit Chow and given tap water *ad lib* for at least one week prio to study. Total thyroidectomies were per formed following administration of veterinary Nembutal. The thyroid tissue was placed i sterile Dulbecco's phosphate buffered salin containing potassium penicillin G (100 units/ ml) and in streptomycin sulfate (100 μg/ml) The thyroid tissue was immediately dissecte free of surrounding connective tissue an thinly cut, using micro dissecting scissors The method of organ culture was that o Fell and Robinson(6) and Trowell(7) wit modifications as previously described(8) ex cept thermally inactivated rabbit serum wa employed in place of human serum. Dupli cate cultures were performed on each tissu studied. As a control for each tissue, methi mazole (70 μg/ml) was added to the cultur medium of one of the duplicate cultures t prevent active incorporation of iodine int protein-bound forms by the thyroid tissue.

Tissue-culture medium 199(9) containin 10% of thermally inactivated rabbit serun was employed at pH 7.4. Carrier-free I-13 was added to this culture medium to yiel

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radioactivity levels of approximately 2 μc/ml.

In one organ culture experiment, thyroid-stimulating hormone (bovine NIH-TSH-B3)† was added to the culture medium in one of the duplicate sets to provide a concentration of 0.2 U per ml. The solution of the TSH was sterilized by passage through a Millipore filter with an average porosity of 0.46 U.

Following organ culture for 24 hours at 37°C, the thyroid tissue was minced and incubated with a 2.5 mg% solution of papain. The culture medium was extracted three times with Butanol and chromatographed. Papain-solubilized samples of the thyroid tissue were also chromatographed in parallel in n-butyl alcohol : 2 N acetic acid (1:1, v/v) and n-butyl alcohol : 2 N ammonium hydroxide (4:5, v/v) systems using the descending technique. Following chromatography, the strips were scanned in a strip scanner (Nuclear-Chicago Actigraph II) and the areas under the radioactive peaks were identified and quantified by planimetry. Location of labeled compounds was achieved by addition of carrier iodoamino acids and stable KI.

The method of tissue culture of isolated thyroid cells was that of Irvine(10) with modifications as previously described(11). Cells were obtained by trypsinization at pH 8.0 after trimming, mincing, and washing of the thyroid tissue. The trypsinizing fluid consisted of 0.25% trypsin in isotonic saline. The trypsinized cells were suspended in 100 volumes of tissue-culture medium 199 which contained 10% thermally inactivated rabbit serum at pH 7.4. Carrier-free I-131 was added to the culture medium to yield radioactivity levels of approximately 2 μc/ml together with 0.02 μg KI per ml of medium to insure constant iodide levels. As a control, portions of the cell suspension were incubated with methimazole (0.02 M).

Following incubation for 24 hours at 37°C, the culture medium was decanted and the cells gently washed with 10% KI to remove any remaining culture medium and also to remove any absorbed I-131 from the surface of the cells prior to protein hydrolysis. Samples were then chromatographed, scanned, and identified as described above.

Three rabbits were given 0.5 millicurie of carrier-free I-131 intravenously. Thyroidectomies were performed 24 hours later. The thyroid tissue was minced and papain solution was added as described previously to accomplish protein hydrolysis. Samples were chromatographed, scanned, and identified.

Uniformly labeled C^{14} tyrosine and C^{14} phenylalanine were obtained from New England Nuclear Corp. with specific activities of 0.05 millicurie per 0.024 mg and 0.05 millicurie per 0.021 mg, respectively. The purity of each amino acid was demonstrated by paper chromatography. The following studies were performed using these labeled amino acids:

1. Rabbit thyroids were incubated in organ culture for 24 and 48 hours with 5 μc of C^{14} phenylalanine per 3.5 ml of tissue culture medium 199 containing 10% thermally inactivated rabbit serum. KI, 0.02 μg, was added to each ml of medium to insure constant iodide levels. At the end of each incubation period, the tissue was removed from the grid and washed with 10% phenylalanine to remove any remaining culture medium and any absorbed C^{14} phenylalanine from the surface of the tissue prior to protein hydrolysis. 2. Rabbit thyroids were incubated in organ culture for 24 and 48 hours with 5 μc of C^{14} tyrosine per 3.5 ml of tissue culture medium 199 containing 10% thermally inactivated rabbit serum. Tyrosine-free tissue culture 199 obtained from Grand Island Biological Co. was used. KI, 0.02 μg, was added to each ml of medium as described above. At the end of each incubation period the tissue was washed with 10% tyrosine prior to protein hydrolysis. 3. Each experiment with C^{14} tyrosine and C^{14} phenylalanine was repeated with addition of thyroid stimulating hormone (Bovine NIH-TSH-B3), 0.2 u per ml of culture medium. 4. Following organ culture for 24 and 48 hours, the thyroid tissues were minced, incubated with papain, and chromatographed. The culture media were extracted 3 times with butanol and chromatographed. Following chromatography, the strips were scanned in a strip-scanner and the areas under the radioactive peaks were

† Kindly supplied by Endocrinology Study Section, Pituitary Hormone Distribution Program, Nat. Inst. Health.

TABLE I. Chromatographic Analysis of Rabbit Thyroid Tissue and Culture Medium After 24 Hr Incubation (% of Total Radioactivity).

		Origin	I-131	MIT	DIT	T_4
		In vivo labeling				
Normal	24 hr	11.2	16.2	32.4	26.3	13.9
		Cell culture				
	24 "	37.9	46.6	6.8	2.4	6.8
		Organ culture				
	24 "	14.3	33.1	31.7	9.8	11.1
		Organ culture media				
	24 "	0	100	0	0	0
		Organ culture				
With TSH	24 hr	9.8	29.7	20.2	21.2	19.1
		Organ culture media				
	24 "	0	95.8	0	0	4.2

identified and quantified.

Portions of thyroid tissue were obtained at the end of all organ culture experiments, fixed in 10% neutral formalin, embedded in paraffin wax, sectioned and strained with H & E.

Results. The results of the analysis of the papained produced hydrolysis of rabbit thyroid glands labeled *in vivo* with I-131, incubated with I-131 in tissue and organ culture and butanol extracts of the organ culture media are listed in Table I. Monoiodotyrosine (MIT), diiodotyrosin (DIT), and thyroxine (T_4) were detected in the tissues. In the methimazole controlled tissue and organ cultures, at least 90% of the total radioactivity in the tissues was represented by origin material or I-131 labeled iodotyrosines (less than 2%). MIT, DIT, and T_4 were detected in rabbit thyroid after 2 hours incubation in organ culture. Iodothyronine was detected only in the butanol extracts of the organ culture medium containing TSH.

Chromatographic analysis of the papain treated thyroid tissue incubated with C^{14} tyrosine with and without TSH revealed the presence of the labeled tyrosine but no C^{14} labeled iodotyrosines or iodothyronines. Similar findings were obtained with papain treated thyroid tissue which had been incubated with C^{14} phenylalanine in that only phenylalanine was detected. Some C^{14} label was incorporated into origin material in all experiments. No C^{14} labeled iodotyrosines or iodothyronines were detected in any of the culture media.

Histological evidence of viability was observed in all of the thyroid explants.

Discussion. The results presented in Table I indicate clearly that histologically normal rabbit thyroid in organ culture concentrates I-131 from the media and labels tyrosine and thyronine. The addition of TSH to these cultures increased significantly the proportion of DIT and T_4 found in the tissues and effected the release of I-131 labeled T_4 in to the culture medium. These effects of TSH are similar to those previously reported by us for organ cultured human thyroids(8).

Our results do not agree with previously published data concerning the metabolism of labeled iodide in rabbit thyroid tissue *in vitro* (1). We believe that the differences in results obtained by us are due to the method of maintaining viability of the thyroid tissue and are not due to a species difference in iodothyronine formation as has been suggested previously(1).

Dobyns(2) found that when C^{14} tyrosine was added to the media of rabbit thyroid slices, some C^{14} was incorporated into iodothyronine but not iodotyrosine. Dillard *et al* (3,4) were unable to demonstrate incorporation of C^{14} tyrosine into iodothyronines when beef and porcine thyroid slices and rabbit thyroid homogenates were incubated *in vitro* with C^{14} tyrosine. Mosier(1) was unable to detect incorporation of the C^{14} label into iodothyronine from media containing C^{14} MIT, C^{14} tyrosine, and C^{14} I-131-DIT. Munez *et al*(5) were also unable to demonstrate incorporation of tritiated tyrosine into iodotyrosines by sheep slices. Most recently, Nataf *et al*(12) reported that fetal rat thyroid in organ culture did not incorporate C^{14} leucine in increased amounts into protein in the presence of TSH. This lack of effect of TSH upon amino acid incorporation was in contrast to the marked increase in I-131 labeling of tyrosine produced by TSH.

Using histologically normal rabbit thyroid which has been shown to both be able to generate I-131 labeled tyrosine and thyronine and to be responsive to TSH administration, we were unable to obtain any evidence for

the incorporation of C^{14} tyrosine into formed thyroid hormones or its precursors. Perhaps phenylalanine might be the precursor of the tyrosine residues of thyroglobulin which eventually become iodinated. We were unable to find any evidence of C^{14} phenylalanine incorporation into iodotyrosine or thyroid hormones either. It is clear, however, that both C^{14} labeled aromatic amino acids were concentrated and incorporated into proteins within the organ cultured thyroid gland; *i.e.;* C^{14} labeled material was found at the origin of our chromatograms as well as the free radioactive amino acids themselves. This latter free material could have been derived from peptide linked amino acids which had been digested free by papain.

Thus we are faced with an apparent anomaly: Histologically and (apparently) metabolically normal thyroid tissue does not incorporate C^{14} labeled tyrosine or phenylalanine into thyroglobulin where it can be first, iodinated and then, in turn coupled to form thyroid hormones.

Two possible explanations could be offered in an attempt to resolve this anomaly:

1. These cultured glands are not making thyroglobulin. The formation of iodotyrosine and iodothyronine and the effects of TSH upon these processes do *not* constitute evidence for thyroglobulin synthesis since iodination and TSH effects can and do occur exclusive of protein synthesis on preformed thyroglobulin(12-15).

2. The specific activity of C^{14} labeled tyrosine or phenylalanine is too low and the amount incorporated into thyroglobulin which is iodinated and/or coupled is too small to be detected by our methodology. The fact that I-131 labeling of tyrosine and thyronine can be demonstrated is not relevant to this explanation since I-131 is used in a carrier-free form with a consequent extremely high specific activity.

Explanation No. 1 would seem unlikely because it has been shown that thyroid slices will rapidly incorporate C^{14} tyrosine into thyroglobulin(16). Therefore it would appear likely that the amount of thyroglobulin tyrosine iodinated and converted to thyroid hormones is a small percentage of the total amount of tyrosine incorporated into all the various thyroid proteins including thyroglobulin. Under these conditions then, the specific activity of iodotyrosines and iodothyronines with respect to C^{14} would be too small for us to measure. Experiments are currently underway to isolate the thyroglobulin from thyroid glands organ-cultured in the presence of C^{14} tyrosine.

Summary and conclusions. 1. Organ cultured rabbit thyroid glands are able to concentrate I-131 from culture media and can incorporate the iodide into MIT, DIT, and T_4. 2. TSH accelerates all of these processes in organ cultured rabbit thyroid glands. 3. No evidence for the incorporation of C^{14} tyrosine or C^{14} phenylalanine into MIT, DIT, and T_4 could be found in histologically normal and apparently metabolically normal organ cultured thyroid glands. 4. The most probable explanation for this lack of incorporation of C^{14} phenylalanine and C^{14} tyrosine is that only a small proportion of tyrosine incorporation into thyroid protein is found as residues of thyroglobulin appropriate for iodination and generation into thyroid hormones. Thus the specific activity of the radioactive tyrosine in MIT, DIT, and T_4 would be too low to measure in spite of culturing in tyrosine-free media.

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Tritiated-Thymidine Incorporating Cells in the Peripheral Blood of Normal and Splenectomized Rats.* (32160)

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An intimate relationship exists between the spleen and the circulating blood cells of mammals. For example, following splenectomy, changes have been reported in the structure and numbers of circulating erythrocytes and there is a sustained increase in platelet count and peripheral leukocyte numbers(1,2). In rats, splenectomy induces a granulocytic and mononuclear leukocytosis(3,4), and elevation in platelet counts(5,6), but peripheral erythrocyte numbers remain unchanged(4,7).

In the present investigation, the proliferative potential of peripheral mononuclear leukocytes from normal and splenectomized rats was studied. It was found that tritiated-thymidine (H^3T)-incorporating cells were present in small numbers in the peripheral blood of normal rats and that splenectomy caused a significant increase in the numbers of these cells in the circulation.

Materials and methods. Seventy-five male rats (200-250 g) of a modified Long-Evans strain, free of *Bartonella muris,* were maintained on Purina chow and tap water *ad libitum* was used. These were divided into 3 groups. The first group (42 rats) was splenectomized as described by Farris and Griffith(8). The second group (21 rats) was sham-splenectomized by carefully exteriorizing and then returning the spleen to the peritoneal cavity and the third group (12 rats) comprised unoperated controls. On post-operative days 1-4, 7, 10 and 23, 6 splenectomized and 3 sham-operated rats were exsanguinated by aortic puncture and total white cell counts and blood smears were prepared from the blood of each rat. Tritiated thymidine (2 μc/ml whole blood; Schwarz BioResearch, Sp. Act. 15.0 c/mMole) was added to the heparinized blood (Heparin Sodium, Fisher, 100 units/ml whole blood), collected from each rat and the mixture incubated (with constant agitation) at 37°C for 1 hour. Each sample was then centrifuged 5 minutes at 1200 rpm. Most of the supernatant was removed without disturbing the buffy coat cells which were resuspended in residual supernatant with Pasteur pipettes to give a uniform cell suspension. Smears made from each suspension were air dried, fixed in methanol, processed autoradiographically and stained as described by Monette *et al*(9). Exposure time was 12 days at 5°C. Control blood samples were treated identically. Labeling incidences were determined by counting 5000 peripheral mononuclear cells for each animal. From this and the total white cell count, the absolute numbers of labeled cells per mm^3 of blood was calculated.

Results. No mortality or anemia developed in the splenectomized rats indicating the absence of *Bartonella muris* infection. The absolute numbers of H^3T-incorporating cells in the circulation of splenectomized, sham-splenectomized and normal intact rats are indicated in Fig. 1. It may be seen that small numbers of mononuclear cells in the peripheral blood of normal rats were capable of incorporating H^3T. One week after sham-splenectomy there was a 2-fold increase in

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