GROWTH OF THYROID TISSUE IN THE GILLS OF AMBLYSTOMA PUNCTATUM REARED IN PROPYLTHIOURACIL

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NINE FIGURES

Antithyroid compounds affect amphibians and mammals similarly in many respects. In both forms, these drugs inhibit the synthesis of thyroid hormone with a consequent increase in thyrotropic hormone from the anterior hypophysis leading to hypertrophy, hyperplasia, and hyperemia of the thyroid gland. Some of the other antithyroid drug effects are related particularly to the nature of the species. The latter effects include the inhibition of metamorphosis in amphibians and a more obvious diminution of melanin in amphibians than in mammals. In a previous study of the effects of propylthiouracil on melanin formation in Amblystoma punctatum, it was noted that animals reared in the compound for several months often developed large cartilage tumors (Copenhaver and Detwiler, '51). These cartilage growths may be an example of a dissimilar antithyroid drug effect for amphibians and mammals. As far as we are aware similar cartilage tumors have not been reported for mammals treated with antithyroid compounds but neither have they been reported by other investigators for amphibians. Consequently, it seemed desirable to repeat and extend the studies on the relationship of propylthiouracil to cartilage tumors in Amblystoma. In the course of these experiments, it was noted incidentally that the gills of a high percentage of the animals contained nodules of thyroid tissue. This finding seemed

to warrant a further study which forms the basis of the present report.

Antithyroid drugs commonly have a tumorigenic effect on the thyroid to the extent that they lead to a pronounced hypertrophy and hyperplasia of the thyroid tissue in its normal location. It is more unusual to find thyroid tissue in an abnormal location. As far as we are aware this result has not been reported previously for amphibians reared in thiouracil and related compounds. In mammals, the growth of thyroid tissue in an atypical location (in the lungs) was noted by Morris, Dubnick and Dalton ('46) for mice which received thiouracil in their diet for 11 months or more. Gorbman ('46) also noted the presence of "thyroid-like cells" in the lungs of mice fed thiourea for a long period. A more detailed report on the growth of thyroid tissue within the lungs of mice fed on thiouracil was given by Dalton, Morris and Dubnick in 1948. They considered the lung thyroid nodules as "benign metastasizing" thyroid tissue rather than "thyroid rests". They inclined to the view of Gorbman ('46) that thyroid cells reach the lung from the hyperplastic thyroid by way of the blood vessels. There are apparently a number of similarities between the lung thyroid nodules of mice and the gill thyroid nodules of Amblystoma. This will be discussed further after a presentation of the data on the growth and transplantability of the gill tumors of Amblystoma.

MATERIAL AND METHODS

Embryos of Amblystoma punctatum were obtained from several different localities over a period of several different seasons. Therefore the results cannot be attributed to the susceptibility of a particular strain of Amblystoma punctatum. On the other hand, a species difference in tendency for tumor formation may exist inasmuch as a limited number of experiments on Amblystoma tigrinum have failed thus far to produce any gill thyroid nodules.

Different series of tests were begun on embryos at different ages ranging from tail bud stages (Harrison's stage 27)

to stages following perforation of the oral ectoderm (Harrison's stage 43). These stages precede the time of cytological and functional differentiation of the thyroid gland. In most cases, the animals were reared continuously in the drug from the embryonic stages until the experiment was terminated either by fixation or by survival limitations of the animals. Some animals have survived for more than 28 months in the drug.

The chemical used was 6-n-propyl-2-thiouracil which was kindly supplied by the Lederle Laboratories Division of the American Cyanamid Company. The drug was made up in spring water in concentrations ranging from 0.01% to 0.005%. The more concentrated solution was used consistently for the first three months of the experiment. The more dilute solution was used at intervals during the latter part of the experiment, whenever the animals became particularly sluggish in their reactions. The animals were reared singly, in finger bowls containing 100 cm^3 of the solution. The animals were transferred to fresh solutions at intervals of approximately 10 days. Control animals were reared in a corresponding amount of spring water. All animals were fed on Daphnia for about 10 days following the yolk resorption stage and chiefly on Enchytraeus for the remainder of the experiment.

Transplantation experiments on gill thyroid tumors were done as follows. Donors and recipients were narcotised in chloretone, 1:2000. Iridectomy scissors were used to make a slit incision through the skin on one side of the dorsal fin of the recipient just caudal to the level of the hind limbs. A subcutaneous tissue space was made in a cephalic direction from the incision by probing with one blade of the scissors. Then a small fragment of tissue from a thyroid nodule previously biopsied from the donor was inserted through the slit incision and pushed forward into the subcutaneous tissue space.

The basal portion of the dorsal fin near the hind limb level is a particularly favorable location for implantation ex-

periments. There is ample space in a rather loose type of subcutaneous connective tissue, the graft becomes vascularized quickly, and it can be observed post-operatively through the translucent skin of the dorsal fin. Some preliminary experiments with implants at the forelimb level and at various levels on the flank were less satisfactory.

Tissues were preserved for histological study by one of the following fixatives: Bouin's fluid, Helly-Maximow, and Champy's fluid. Serial sections were stained with one of the following combinations: hematoxylin and eosin, hematoxylin and azure II-eosin, Masson, and periodic acid-Schiff.

OBSERVATIONS

Formation of thyroid nodules on the gills. Tumors began to develop on the gills of some animals as early as 6 months after the start of propylthiouracil treatment. With prolonged immersion in the drug, the percentage of animals with tumors increased and the number of tumors per animal increased also. In a series in which embryos were started in the drug in April of 1954, 13 out of 20 animals had gill tumors after approximately 7 months. The time when the gill tumors were first observed in these 13 cases ranged from 186 days to 222 days of treatment with an average of 196 days. This series of animals is still in the antithyroid drug and it is probable that the percentage of tumor-bearing animals will increase with time. In the 1953 series, with a duration of 496 days, gill tumors were present in 12 out of 16 animals. In the 1952 series, with some animals in the drug for 722 days, gill tumors developed in 19 out of 23 animals.

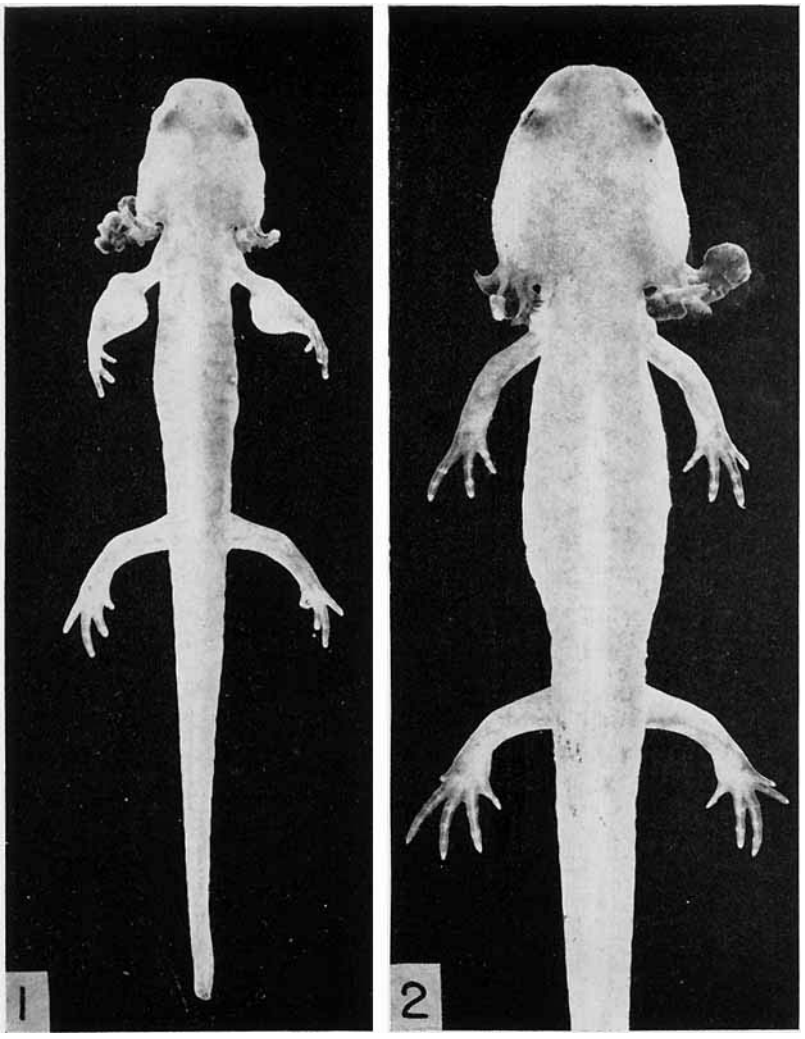
There was a tendency for the gill tumors to appear at about the time when pair-fed controls were completing metamorphosis. In the 1954 series cited above with thyroid tumors in 13 out of 20 animals by 7 months, the tumors were first observed on animals which ranged in size from 42 to 57 mm. Pair-fed controls from the same egg clutch metamorphosed at lengths ranging from 40 to 48 mm.

Gill thyroid tumors tended to appear later than leg cartilage tumors. In the 1954 series, there were leg cartilage tumors in 17 out of 20 animals after 7 months. Lengths of animals at the time of initial development of cartilage tumors ranged from 29 mm to 53 mm, with an average of 42 mm, in contrast to an average length of 47 mm for the same series of animals at the time of appearance of gill tumors. In regard to age, the cartilage tumors developed when the duration of the drug treatment ranged from 91 to 222 days, with an average of 158 days in contrast to an average of 196 days for thyroid tumor appearance in the same series.

Since each of the types of tumors (thyroid and cartilage) appeared on more than 50% of the animals, the frequent occurrence of both types on the same individual, as shown in figure 1, was not an indication that one type of tumor is dependent on the other. In the total series of cases extending over 4 years (1951–54), there have been a number of individuals with only one type of tumor as illustrated in figure 2.

The gill tumors began as small nodules on a gill filament, usually toward the distal end of the gill. The initial tumors frequently appeared on the second pair of gills but sufficient data have not been collected on this point to determine whether the predilection for the second gills is statistically significant. With increased time in the drug, the initial tumors increased in size and additional tumors formed, frequently appearing on each of the three pairs of external gills.

Grossly, the gill thyroid nodules varied from a pearly gray color in some cases to a deep red in others. Microscopically, the gill thyroid nodules resembled the normally-located thyroid tissue of propylthiouracil treated animals with the exception that the para-follicular blood vessels were generally less enlarged in the gill nodules. Limitations of para-follicular space may have been a factor in this. The gill nodules were composed of thyroid follicles lined by an epithelium ranging from low cuboidal to high columnar, with the latter type predominating (figs. 3 to 5). Numerous mitotic figures and numerous bud-like outpouchings from the



Figs. 1 and 2 Photographs of Amblystoma punctatum larvae reared in propylthiouracil. Figure 1, animal reared in the drug for 593 days, showing gill thyroid tumors and leg cartilage tumors concomitantly; figure 2, animal reared in the drug for 383 days, showing gill tumors in the absence of leg tumors. Both animals show a reduction in pigmentation. Length of animals: 78 mm, and 72 mm, respectively; photographed at × 1.5 and × 2.4 respectively.

follicles indicated active hyperplasia. In a number of cases there was evidence of intrafollicular hemorrhage.

Although the amount of colloid varied in different thyroid follicles within the same gill nodule, there appeared to be a distinctive difference for different animals. In some cases, the nodules were composed chiefly of large, cystic-like follicles filled with colloid (fig. 3). In other cases, the nodules were composed primarily of irregularly-shaped follicles with less colloid (fig. 4). The colloid of the gill tumors, like that of the thyroid itself, apparently lacked thyroid hormone since none of the animals metamorphosed while they were reared in propylthiouracil. They did metamorphose when they were transferred to water.

Transplantation of gill thyroid nodules to young larvae reared in water. Nine experiments were made in which a fragment of gill thyroid tumor was transplanted to the dorsal fin as described in the section on material and methods. The recipients were larvae at Harrison's stage 41 (about 4 days before the yolk resorption stage). They were reared in spring water and fixed for histological study at intervals ranging from three to 82 days after operation and at lengths ranging from 16 mm to 47 mm. At the latter stage they were approaching metamorphosis.

Observations of the tumor region with the aid of a binocular microscope at intervals during the course of the experiment indicated a lack of growth in the transplanted tumor. Histological studies of sections showed that thyroid follicles with variable amounts of colloid were present for the duration of the experiment, but there was little evidence of growth.

Sections from a sample of the donor tissue at the time of operation indicated that colloid was relatively scanty in this particular tumor and that the follicular cells were typically tall columnar. Transplants fixed at 3, 18 and 32 days respectively after operation showed a gradual change from a columnar to a cuboidal epithelium. Pycnotic nuclei, indicative of degeneration, were scarce. At 52 and 82 days postoperatively, the follicular epithelium varied from cuboidal to

squamous and was generally lower than normal for larval Amblystoma. However, the colloid was at least as abundant as in the original tumor. Although the transplants failed to increase in size for the duration of the experiment, they maintained themselves and the alteration of their epithelium may have been due to a functional change.

Transplantation of gill thyroid nodules to older larvae reared in water. These experiments were similar to those just described with the exception that the recipients were larvae about 40 mm in length. In spite of the different age of the recipients, the results were generally similar to those described above. The experiment was terminated at 92 days after operation (58 days after metamorphosis of the host) because observations on the living animals failed to show any growth of the graft. However, sections showed the persistence of small grafts with low cuboidal epithelium and with some colloid.

Transplantation of gill thyroid nodules to larvae reared in propylthiouracil. Six experiments were done in which fragments of gill nodules were grafted to larvae reared continuously in propylthiouracil, including 236 days in the drug prior to operation. A definite growth of the graft was observed grossly in this series and the recipients were kept for a longer period than in the previous series, — they were fixed at intervals ranging from 28 to 281 days after operation.

Microscopically, the transplanted tumors retained the general appearance of the donor gill thyroid tissue. They also resembled the hypertrophied thyroid gland of the donor animal rather than the gland of a normal larva (see figs. 6 to 9). Numerous mitotic figures and frequent bud-like protrusions from follicles into surrounding tissues indicated active growth of the transplants. As in the gill nodules, the para-follicular blood vessels were not as enlarged as those of the hypertrophied thyroid in situ. The height of the follicular epithelium varied from low cuboidal to tall columnar for different follicles. In some cases, there was evidence of intrafollicular hemorrhage.

DISCUSSION

In a previous study of the effects of prophylthiouracil on Amblystoma embryos, it was found that the antithyroid drug in concentrations of 0.01% had no significant effect on growth rate preceding the yolk resorption stage but that it retarded growth during later larval life (Copenhaver and Detwiler, '51). It was suggested that the reduced growth rate was related chiefly to depressed food intake as described by Leathem ('46) for rats fed thiourea. The difference between the growth rates of normal A. punctatum larvae and propylthiouracil-treated animals was most pronounced when all of the animals were offered all the food they would eat, commonly called "maximal feeding." When the amount of food offered did not exceed the maximum appetite of the experimental animals, the controls were underfed and the difference between the growth rates of experimental and control animals tended to disappear. In practice, it was found that the propylthiouracil-treated animals remained healthier when they were given even less food than their subnormal choice. Consequently, the feeding in these experiments was considerably less than "maximal" for the controls. The latter were pair-fed until they grew at approximately the same rate as the experimental animals.

A question arose whether markedly subnormal feeding would in itself produce a change or "stress" leading to the formation of tumors of cartilage and thyroid. To test this point, Amblystoma larvae growing in water were given as little food as seemed compatible with normal survival. The underfed animals metamorphosed at 8 months after the yolk resorption stage, at an average length of 43 mm whereas animals fed maximally metamorphosed in 8 weeks, at an average length of 47 mm. Thus far, no amount of subnormal feeding has produced any tumors. Furthermore, the spontaneous development of these tumors has never been observed in any of our series of animals reared in water.

It has been noted that the gill tumors frequently develop at about the time when pair-fed controls metamorphose. It

is known that the pituitary gland of normal animals increases its output of thyrotropic hormone preceding metamorphosis but it is not known whether there is a similar change at this age in propylthiouracil-treated animals in which there is already an accelerated production of thyrotropic hormone. Studies are being continued to determine whether there are detectable cytological changes in the anterior hypophysis at the particular time when the gill tumors arise. In other studies on the rôle of the hypophysis in the growth of gill tumors and leg cartilage tumors, hypophysectomized animals are being reared for an extended period in propylthiouracil.

There is a question concerning the origin of the thyroid tumors on the gills. Are they embryonic "rests" stimulated to growth by thyrotropic hormone after prolonged exposure to the antithyroid drug? Since the thyroid primordium arises from entoderm closely associated with the pharyngeal pouches it is conceivable that thyroid remnants might exist in the gills as they do on occasion in the pericardium and myocardium of mammals. However, we have never observed such remnants in the gills of normal Amblystoma, nor have we found any thyroid tissue within the heart in our series of studies on Amblystoma. Furthermore, it is unlikely that "thyroid rests" would occur in 80% of the animals as did the thyroid tumors in our 1952 series of propylthioracil-treated animals. In one case a thyroid tumor occurred on the hind limb where it obviously could not be explained as a "thyroid rest." We are inclined to believe that thyroid cells enter the enlarged venous channels of the hyperplastic thyroid gland to be carried through the heart to the gill capillaries where they are filtered out and establish thyroid growths. This is similar to the explanation given for thyroid tissue within the lungs of thiouracil-fed mice (Gorbman, '46; Dalton, Morris and Dubnick, '48). Reference to figure 7 shows how thyroid follicles protrude into the enlarged vessels to such an extent that they might readily become detached.

The thyroid tissue of the gill nodules generally resembled that of the host thyroid. When the intact thyroid contained

more colloid, the gill thyroid nodules also contained more. Hypertrophy and hyperplasia of follicular epithelium usually persisted both in the host thyroid and in the tumor, although these were often more pronounced in the tumor. Regression in histological activity in the thyroid gland after prolonged exposure to antithyroid drugs appeared to be less for Amblystoma than that described by Gordon, Goldsmith and Charipper ('45) for Rana pipiens. Different species appear to differ in their regression from the effects of antithyroid drugs just as different species vary in their rate and degree of initial response as described by Astwood ('45).

The results with transplantation of gill thyroid tumors in Amblystoma differ in only a few details from those described for lung thyroid tumors in mice. In the latter species, transplants to normal animals do not take but transplants to thiouracil-fed hosts grow rapidly. In Amblystoma, transplants to normal animals take in the sense that they maintain themselves for a long period but they show very little growth whereas transplants to propylthiouracil-reared animals grow rapidly. Since practically all tissues are more transplantible in amphibians than in mammals it is not surprising to find slightly different results for amphibians and mammals in the case of transplants to normals. The general difference between the results for normal and antithyroid-treated animals were the same.

In mice, reimplantation of a strain of lung thyroid tumor to 7 successive generations of thiouracil-fed hosts has produced a "potentially malignant" tumor which grows on normal animals (Morris, Dalton and Green, '51). Amblystoma gill thyroid tumors should be retransplanted to successive generations of propylthiouracil-reared animals to determine whether a malignant type of thyroid tumor can be developed for this species.

SUMMARY

1. This study deals with the growth of thyroid tissue on the gills of Amblystoma punctatum reared in solutions of

propylthiouracil ranging from 0.01% to 0.005%. The drug treatment began when the animals were tail-bud embryos and continued until they were larvae more than two years old.

2. Gill thyroid tumors began to appear on some animals after 6 months exposure to the drug; they were present on more than 50% of the animals after 8 months, and they increased in number and size with increased duration of the treatment. In one case, a thyroid tumor formed on the hind limb.

3. Microscopically, the gill tumors were composed of thyroid follicles resembling those of the intact thyroids. The follicular epithelium was hypertrophic and hyperplastic, often more so than that of the thyroid gland itself. Colloid was present in variable amounts. Para-follicular blood vessels were not as enlarged as in the host thyroids.

4. Transplants of gill thyroid tumors to the tail of propylthiouracil-reared animals showed marked growth whereas similar transplants to normal animals persisted with only slight growth. In some respects, the gill thyroid tumors of propylthiouracil-reared Amblystoma resembled the lung thyroid tumors of thiouracil-fed mice described by Dalton, Morris and Dubnick ('48).

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PLATE 1

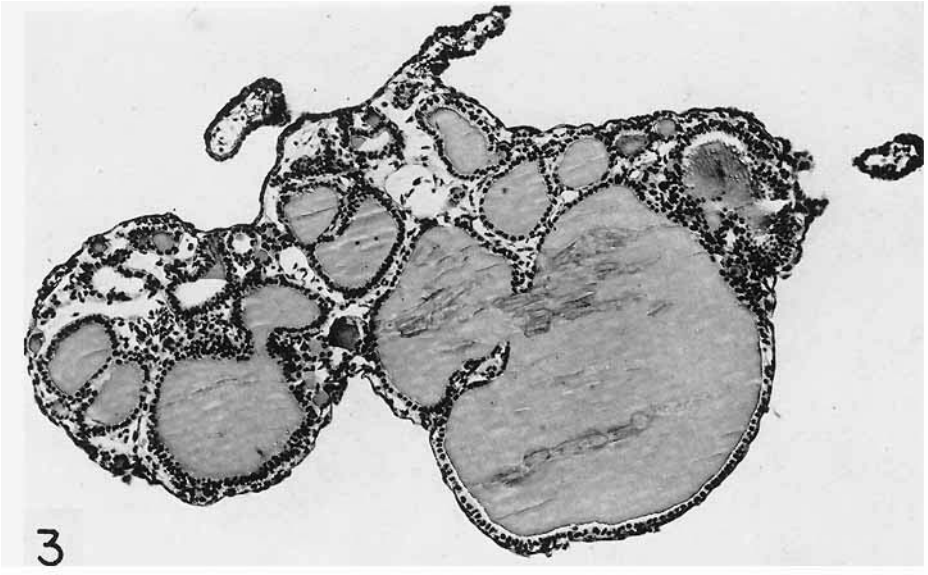
EXPLANATION OF FIGURES

Figs. 3 to 5 Photomicrographs of cross sections of the gills of Amblystoma punctatum larvae reared in propylthiouracil.

3 Unusually large thyroid follicles in the gills of an animal reared in the drug for 523 days. A few gill filaments are shown above and at the right but most of them disappeared during the growth of the tumor. × 56.

4 Typical structure of gill thyroid tumor with follicles of different sizes and shapes in an animal reared in the drug for 588 days. Note that many of the follicles are in contact with the thin epithelial covering of the gill. × 56.

5 Higher magnification showing follicular epithelium in a gill tumor from an animal reared in the drug for 253 days. A few follicles show evidence of intrafollicular hemorrhage. × 330.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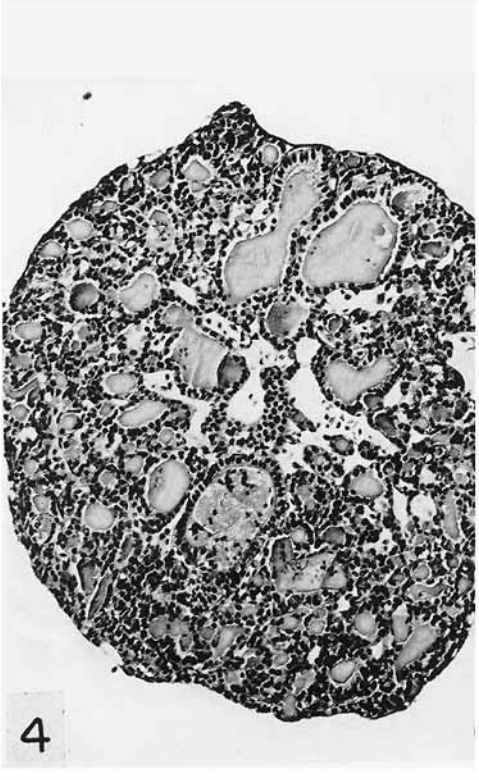
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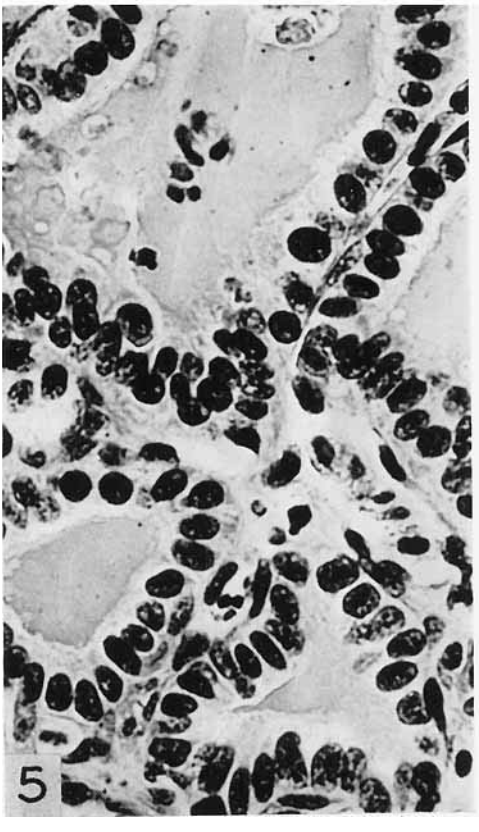
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PLATE 2

EXPLANATION OF FIGURES

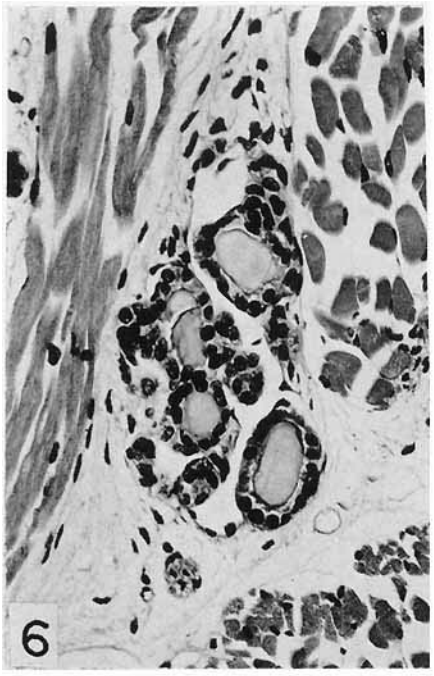
Figs. 6 to 9 Photomicrographs of sections which show similarities in thyroid tissue taken from a gill tumor transplant, a gill tumor, and the intact gland of a propylthiouracil-reared animal in contrast with the thyroid of a pair-fed control.

6 Thyroid follicles of normal size, with cuboidal epithelium, from a 45 mm length pair-fed control which was in the process of metamorphosis 253 days after the yolk resorption stage. Note normal size of para-follicular blood vessels which happen to be empty in this particular section. Skeletal muscle is seen in the field peripheral to the thyroid. × 166.

7 Hypertrophied follicles with columnar epithelium from the intact gland of a 47 mm length propylthiouracil-treated animal 253 days after the yolk resorption stage. Note enlarged blood vessels which are partially filled with nucleated erythrocytes. The thyroid follicles protrude into the vessels with a mesenteric-like attachment to the vessel wall. × 166.

8 Thyroid tumor on the gills of the animal described in figure 7, same magnification.

9 Thyroid tissue from the tail of the same animal, 32 days after transplantation of a gill tumor from another animal previously reared in propylthiouracil for 652 days. Epidermis is seen above the graft in upper right corner of figure. × 166.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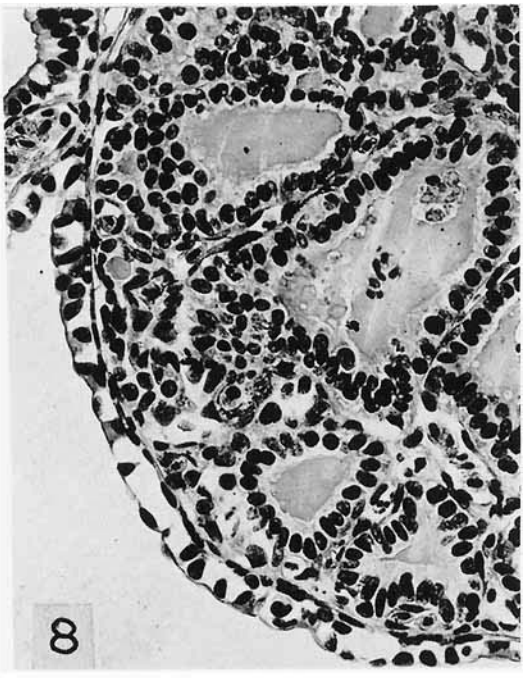
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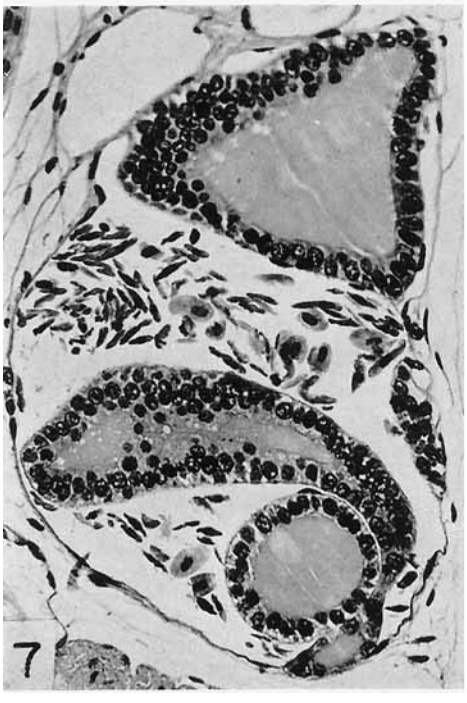
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