STUDIES OF HYPERTHYROIDISM.

III. Bile Pigment Production and Erythrocyte Destruction in Thyroid-Treated Amphibian Larvæ.

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Plate 26.

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Many profound changes, internal as well as external, occur rapidly in amphibian larvæ in which an experimental hyperthyroid condition is induced. During the progress of experiments upon thyroid-fed tadpoles pronounced variations became apparent in the color of the bile. These variations, moreover, were quite regular. Accordingly, special attention was directed to the gall bladder and liver with the object of obtaining data in regard to the change in bile pigment production and the possible significance of this change as an indicator of erythrocyte destruction.

The material used consisted of larvæ of the tree frogs (*Hyla crucifer* and *Hyla versicolor*), of the green frog (*Rana clamitans*), of the toad (*Bufo americanus*), and of the salamander (*Amblystoma punctatum*). These were collected at Woods Hole, Massachusetts, during the latter part of June and early July. Additional green frog tadpoles were collected during the winter at Charlottesville, Virginia. In age and stage of development the animals varied greatly, ranging from 2 weeks after hatching, as in the case of the toad tadpoles, to near the time for metamorphosis, as in some of the tree frog tadpoles. Well developed hind limbs, but no erupted fore limbs, were present in the tadpoles of *Hyla crucifer*, *Hyla versicolor*, and *Rana clamitans*. In one batch of *Hyla crucifer* tadpoles the hind limbs were scarcely visible. In the toad tadpoles the hind limbs were not visible. The salamander larvæ possessed prominent external gills, and both limbs were well developed. Metamorphosis was accelerated and the hyperthyroid condition induced by placing dried sheep thyroid extract in the water with the animals. In a few cases pure thyroxin crystals were used. Animals were killed and the gall

bladders examined at intervals of 1 to 21 days. A few animals were killed and examined during the first 24 hours of treatment. All parts of the body were examined microscopically and a detailed study was made of the liver, spleen, kidney, gut, gills, and tail with particular attention to the conditions of erythrocyte destruction. The Giemsa eosin-azure blood technique was employed. Observations were also made on preparations of living blood.

OBSERVATIONS.

The macroscopic changes observed in *Hyla crucifer* are clear-cut. They are also similar to those observed in the other species studied and may be taken as typical. The normal gall bladder full of bile is pale watery green and quite transparent. The bile itself, when made to stain a piece of white paper, presents a very faint tinge of green. At the end of the 1st day of thyroid treatment a very slight deepening of the green color may sometimes be detected. At the end of the 2nd day the color is definitely a deeper green and this becomes progressively more pronounced in 3, 4, 5, and 6 day old thyroid-treated animals. The normal pale green color of the gall bladder changes to a deeper brighter green, then to an emerald-green, and finally to a dark emerald-green. The bile pigment, biliverdin, apparently becomes increasingly concentrated in the gall bladder. During this period the animal exhibits the typical changes of metamorphosis, being distinctly slimmer and smaller at the end of 2 days, the fore limbs erupting after about 4 days, the gills and tail degenerating, the latter being nearly resorbed at the end of 6 days. In the case of the smaller and more immature animals, metamorphosis proceeds only to a certain critical stage. Metamorphic stasis results, usually followed by death.

In the case of the larvæ of the tree frog (*Hyla versicolor*) the color of the normal full gall bladder is a pale transparent greenish yellow with perhaps more yellow than green. With thyroid treatment the green becomes much deeper. In the case of the green frog tadpole some yellow is also present in the bile. This tadpole, because of its large size, is not so quickly affected by the thyroid administration. The succession of color changes presented by the bile proceeds somewhat more slowly. The larger the animal the longer the latent period required for the thyroid extract to produce its effect. Thus, in this species the bile color changes are most pronounced after from 4 to 20 days following the first thyroid administration. In salamander

larvæ the changes from very pale green to deep green could best be seen after from 4 to 15 days of treatment with thyroid extract.

In all species it was possible to arrange a color series with the pale green, or pale yellowish green normal gall bladders at one end and the dark green of the advanced thyroid-treated animals at the other end, with the intermediate shades of green between, varying in depth according to the length of thyroid treatment. A few observations upon untreated tadpoles undergoing normal metamorphosis show that there is a deepening of bile color. Although quite definite, this is not so pronounced as in the experimental tadpoles undergoing thyroid-accelerated metamorphosis.

TABLE I.

Species.	No. of normal animals.	No. of thyroid-treated animals.
Hyla crucifer	20	20
Hyla versicolor	38	16
Rana clamitans	21	15
Amblystoma punctatum	17	14
Bufo americanus (2 wks. after hatching)	250	300
Bufo americanus (older)	15	40
Total	361	405

Table I shows the number of observations made upon normal and experimental animals in each species.

All normal immature tadpoles possessed pale colored bile with relatively little pigment concentration. All thyroid-treated animals after a latent period of about 2 days exhibited darker colored bile with very marked increase in pigment concentration. No exceptions were seen.

Histological Data.

The Liver.—Some increase in erythrocyte degeneration was evident in the sinuses of the liver in the case of the thyroid-treated animals. This was apparent from the increased number of fragments of erythrocytes and abnormal forms (Figs. 1 to 4 and 7). The erythrocyte fragments often become aggregated in groups, with a tendency for the

fragments to round up into globules. In the later stages of thyroid treatment the number of large mononuclear phagocytic cells (macrophages, monocytes, reticulo-endothelial cells) with ingested erythrocyte débris becomes markedly increased (Figs. 5 and 6). Melanin-like pigment is also prominent in the liver, being handled by the macrophages and often being associated in these cells with hemoglobin débris from worn out erythrocytes.

The Spleen.—The activity of the spleen seems to vary greatly as regards erythrocyte degeneration in different individuals. The histological evidence indicates that there may be some increase in erythrocyte destruction by fragmentation (Figs. 8 and 9). Typical macrophages are present containing ingested erythrocyte fragments and globules (Fig. 10), but a definite increase in the number of these cells in the spleen was not seen. The fact must be taken into account, however, that the sinuses of the spleen are largely drained of their cellular content after the thyroid extract has exerted its effect (1).

The Kidney.—There is almost no erythrocyte destruction in the kidney either of the normal or of the thyroid-treated tadpole.

The Gut.—In many regions throughout the gut of thyroid-treated animals from pharynx to lower intestine, there may be seen many macrophages passing through the lining toward the lumen (Fig. 12). These are especially conspicuous during the later stages of thyroid administration. They contain erythrocyte débris largely. Very active sloughing off of lining cells takes place, and associated with this is a certain amount of extravasation of erythrocytes.

The Gills.—In the degenerating gills of the later thyroid-treated animals may be seen many macrophages filled with ingested erythrocyte fragments (Fig. 11). These seem in many cases to pass through the gill epithelium into the gill chamber, and are thus lost to the body.

The Tail.—In the degenerating tail of thyroid-treated tadpoles during the process of resorption may be seen extravasations of red blood cells.

Fresh Blood Preparations.—Fresh blood mounts were made and examined, the cover-glass being sealed with vaseline. More non-nucleated erythrocyte fragments were apparent in the thyroid-treated tadpoles, both in the liver blood and in the peripheral blood.

INTERPRETATION AND DISCUSSION.

The observations upon the bile color of normal and thyroid-treated tadpoles leaves no doubt but that the hyperthyroid condition brings about greatly increased bile pigment concentration in the gall bladder. Such concentration might be favored by (1) a decrease in the amount of water passed into the bile; (2) resorption of water from the gall bladder; (3) an increase in the rate of bile pigment formation. Each of these possibilities may be considered.

1. It is known that water is extruded from the body of a tadpole during thyroid-accelerated metamorphosis. The tissues are dehydrated to a certain extent. Loss of water from the tissues of the animal as a whole might be reflected in a decreased amount being passed into the bile. To a limited extent, this may account for the deeper bile color that does occur.

2. There is a possibility that water may be resorbed from the gall bladder bile, especially if the bladder becomes somewhat distended. During the first couple of days of thyroid treatment the gut is emptied of its contents, and it then remains during the metamorphosis practically devoid of food. The animal eats little or nothing during this period. With the stimulus of food in the alimentary tube lacking there may be a decrease in the amount of bile passing from the gall bladder to the intestine. This may lead to an accumulation of bile in the gall bladder. If water is then resorbed the bile pigment would become more concentrated. There is no special evidence that this process does take place, but it may be a minor factor in bringing about the pigment concentration in the gall bladder. It hardly seems entirely adequate, however, to account for the extreme changes observed.

3. In all probability the major factor contributing to pigment concentration in the gall bladder bile is a greatly increased rate of elaboration of the pigment in the thyroid-treated animals. The bile pigments, biliverdin and bilirubin (and derivatives), are believed to be formed from hemoglobin. In Rich's recent review of the bile pigment situation (2), the conclusion is reached that hemoglobin furnishes the chief and perhaps the only source of bile pigment. The supply of hemoglobin must come from worn out erythrocytes. There is abundant evidence that erythrocyte destruction proceeds at a greatly accelerated rate in the thyroid-fed tadpoles. This destruction precedes

and accompanies the increased rate of bile pigment production. Strong support is, therefore, afforded for the deduction that this constitutes the chief part of the mechanism by which hyperthyroidism brings about the characteristic color changes in the bile.

That thyroid autacoid should have a destructive effect upon the erythrocytes is not surprising. It is rather to be expected. A rapid change from the relatively tranquil life of the tadpole with low metabolic rate to a quickened existence with high metabolic rate is induced by the thyroid administration. Helff (3) has shown that the oxygen-carbon dioxide exchange is greatly heightened. The circulation is also speeded up. The importance of the erythrocyte in relation to these changes is apparent. The senile erythrocytes are ready for destruction at a greatly increased rate. At the same time large numbers of lymphoid hemoblasts are stimulated to proliferate and to undergo differentiation into young erythrocytes. It is characteristic of thyroid treatment that it effects a rejuvenation process; first, by destruction or elimination of the older and more highly differentiated cells, and second, by stimulation of undifferentiated cells to proliferate and then to differentiate. The destruction of the older erythrocytes represents the first step in the blood cell reorganization. The second step, the stimulation of the undifferentiated bood cells, the hemoblasts and their mesenchymal precursors, has already been considered in detail in an earlier contribution (1). A parallel example is found in the response of epithelial surfaces to thyroid administration. In the skin the outer older layers are sloughed off and eliminated. The basement membrane layer containing the younger less differentiated cells is stimulated to proliferate and produce new cells, which later become more highly differentiated. The same activities are exhibited by the lining of the gut throughout a large part of its extent. The blood cells, therefore, in responding to thyroid administration by destruction of the older types (senile erythrocytes) and by stimulation of the younger types (lymphoid hemoblasts and their mesenchymal predecessors) are not unique, but are like other cells of the body.

The senile erythrocytes often seem to lose their nucleus before fragmenting. This may occur by nuclear extrusion, nuclear resorption, or cytoplasmic segmentation (Figs. 3, 4, 7, 8, and 9). This condition in the frog tadpole is of interest for comparison with the con-

ditions in urodeles. Emmel (4) has pointed out that in many urodele amphibians a small percentage of the red blood cells is normally without a nucleus. These arise by "cytoplasmic segmentation" from the nucleated erythrocytes.

In the frog tadpole the senile cells and fragments are rapidly filtered out from the general circulation, chiefly in the liver and spleen. Here fragmentation and globule formation proceed, followed by ingestion by the macrophages. Phagocytosis of erythrocytic globules and fragments becomes more conspicuous in the later stages of thyroid treatment. Groups of macrophages may finally undergo extensive disintegration, especially in the liver and spleen. Although these cells are probably of importance in the elaboration of the bile pigment, the exact mechanism of the process is not known.

The appearance of the gut is of special interest. Macrophages containing ingested material, some of which is erythrocyte débris, pass in large numbers through the lining of the gut into the lumen (Fig. 12). Presumably they are then egested with the intestinal contents. It is, of course, possible that some of this material may be reabsorbed. Likewise, in the atrophying gill region macrophages containing erythrocyte débris apparently pass through the gill epithelium to the gill chamber and thus leave the body (Fig. 11). This apparent loss to the body of destroyed erythrocytes suggests the conclusion that no special attempt is made to conserve the hemoglobin.

Rous and Robertson (5, 6) in mammals of several species have shown the importance of the method of red blood cell destruction by fragmentation, both in normal animals and in animals rendered either anemic or plethoric. Essentially similar conditions appear to obtain in normal and thyroid-treated tadpoles. Rous and Robertson (6) also find that in animals rendered plethoric or anemic increased destruction of red corpuscles by fragmentation takes place without loss of hemoglobin. In the anemic animals there is no increase in bile pigment output. These results are of interest for comparison with the conditions in thyroid-treated tadpoles. In the tadpoles hemoglobin is excreted at a greatly increased rate, both in the form of bile pigment and in the form of hemoglobin débris contained within the macrophages which migrate through the lining of the gut and through the epithelium of the involuting gills. As the gut wall is itself undergoing

profound involution changes, it is likely that little or no reabsorption of this material occurs. The whole reaction is probably a vicious one, as far as the best interests of the tadpole are concerned. In spite of the fact that thyroid treatment induces a higher metabolic rate, which would seem to require a greater hemoglobin basis for its maintenance, the body apparently gets rid of a part of its hemoglobin very rapidly. Thus it is that the anemic condition results, which is associated with metamorphic stasis, and which may be partly the cause of the usual death in this stage.

Rous and Robertson regard it as probable that the normal fragmentation of the red cells may be a result of the perpetual sieving and squeezing to which they are subjected in the finer capillaries. Under conditions of vigorous exercise Broun (7) has shown that increased blood cell destruction occurs, and Broun, McMaster, and Rous (8) have shown that increased bile pigment output results. In the case of the tadpoles, thyroid administration is followed by increased rate of heart beat, increased circulatory rate, and increased muscular activity. It is clear, therefore, that under these conditions the erythrocytes would be subjected to rougher treatment than under normal conditions. In this way the increase in erythrocyte destruction and fragmentation can be accounted for.

The possible influence of food on bile pigment formation, such as has been claimed by Whipple and Hooper (9), may be ruled out in the experiments on thyroid-treated tadpoles. Animals undergoing rapid thyroid-induced metamorphosis eat practically nothing. Usually they were kept in jars containing no food material except that put up with the thyroid extract. Furthermore, the typical changes occur in animals after the administration of pure thyroxin crystals only.

SUMMARY.

Experimental hyperthyroidism in urodele larvæ (*Amblystoma*) and anuran larvæ (*Rana*, *Bufo*, and *Hyla*) is accompanied by definite changes in bile color. The normal pale green, or pale yellow-green, color of the full gall bladder changes progressively after thyroid administration to a brighter green, then emerald-green, and finally a very dark green. In several hundred observations no exceptions were noted.

The bile pigment, biliverdin (and its derivatives), is elaborated from the hemoglobin of worn out erythrocytes. Thyroid administration induces an increased rate of erythrocyte destruction, and this is followed by an increased output of bile pigment. Other minor factors are mentioned which may to a limited extent modify the color of the bile.

Erythrocyte destruction occurs largely by enucleation, cytoplasmic segmentation, and fragmentation, and is probably widespread in the body. Many fragments and senile red cells collect in the liver. During the later stages of thyroid treatment the macrophages become conspicuously active. They are especially abundant in the liver, the gut, and the gills. In addition to the hemoglobin eliminated after transformation into bile pigment, some is transported by macrophages through the gut lining, and to a less extent through the involuting gill epithelium, and thus eliminated from the body.

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[1] This paper gives an extensive bibliography on the subject of bile pigment.

EXPLANATION OF PLATE 26.

All drawings were made from tissues of a green frog tadpole (*Rana clamitans*). This specimen was killed 15 days after the first thyroid administration and shows all of the typical stages in erythrocyte destruction and removal. The tissues were stained with the Giemsa eosin-azure mixture.

FIG. 1. Two normal erythrocytes. × about 1045.

FIG. 2. Degenerating globules of worn out erythrocytes from liver sinus. × about 1045.

FIG. 3. Two large erythrocyte fragments from liver sinus. × about 1045.

FIG. 4. Several erythrocytic globular fragments and two erythrocytes after nuclear extrusion or resorption, from liver sinus. × about 1045.

FIG. 5. Large mononuclear phagocyte (macrophage, monocyte, reticulo-endothelial cell) from liver sinus, containing one large erythrocyte fragment and many smaller ones. × about 1045.

FIG. 6. Large mononuclear phagocyte from liver sinus, with two ingested hemoglobin-containing globules and a few granules of a melanin-like pigment. × about 1045.

FIG. 7. Erythrocyte from liver sinus apparently about to undergo enucleation. × about 1045.

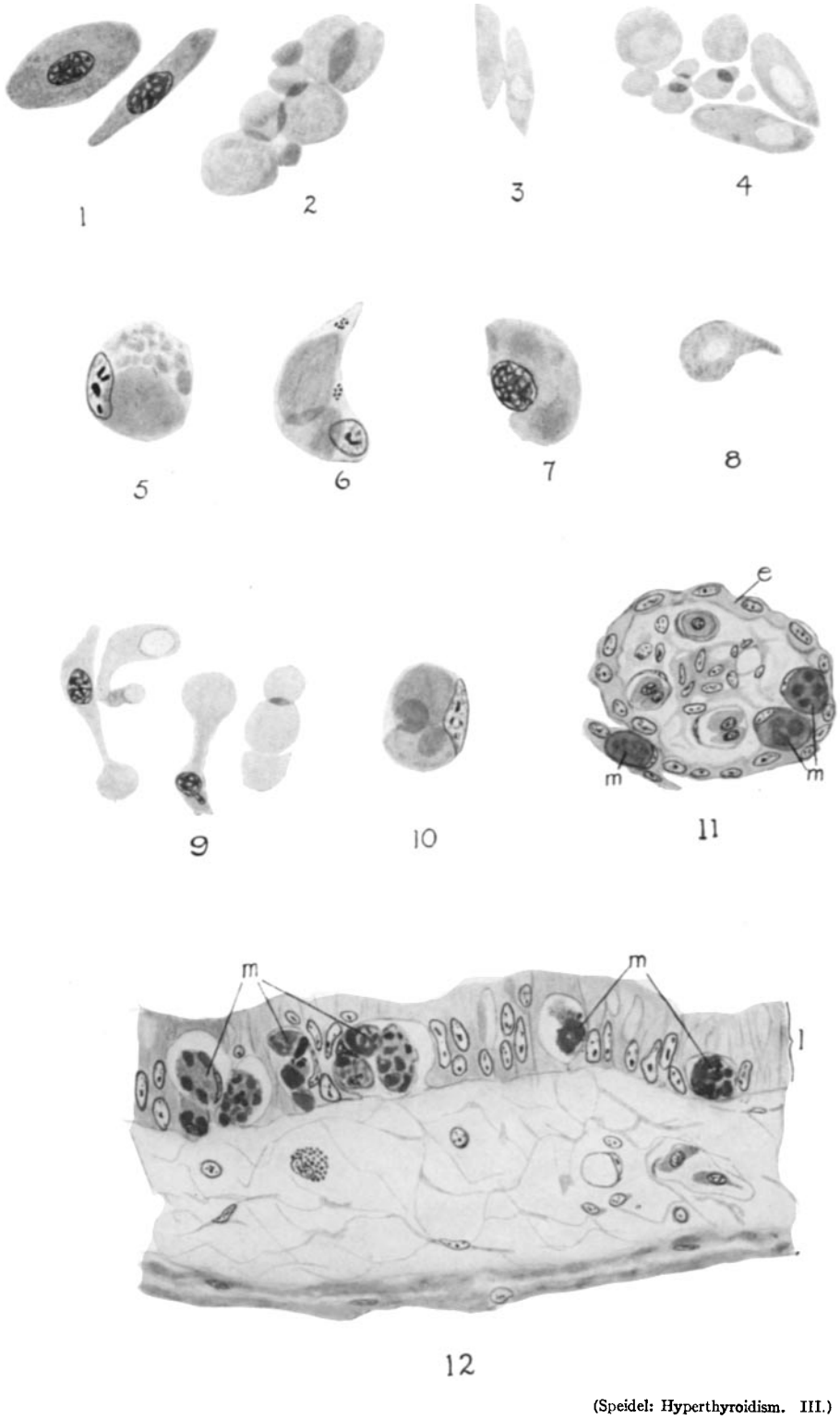
FIG. 8. Enucleated erythrocyte fragment from the spleen. × about 1045.

FIG. 9. Two nucleated erythrocytes from the spleen whose contour suggests one method of globule formation by cytoplasmic segmentation. The second cell from the left represents an enucleated erythrocyte with its cytoplasm at one pole fragmenting into globules. At the extreme right are three hemoglobin-containing globules, possibly derived from a single senile erythrocyte. × about 1045.

FIG. 10. Macrophage from the spleen containing two large and two small erythrocytic globules. × about 1045.

FIG. 11. Cross-section through a gill filament showing three macrophages (*m*) loaded with erythrocyte débris, passing through the gill epithelium (*e*). × about 460.

FIG. 12. Section through the posterior portion of the intestine, showing groups of macrophages (*m*) migrating through the lining (*l*). The macrophages contain other ingested material in addition to the erythrocyte débris. × about 460.



(Speidel: Hyperthyroidism. III.)