Studies on Connective Tissue of the Cock Comb. II. Effect of Androgens.* (22673)

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Although the capon comb test has been used extensively for the biological assay of androgens, relatively little is known of the mechanism of this reaction in terms of tissue structure. The appearance of a so-called mucoid connective tissue has been recognized as the final result of hormone stimulation(1). An increase in the water content of the comb has been observed and is considered to be an important factor in the volume increase; based on this, the term "expansion growth" has been suggested for this reaction(2,3). Proliferation of the connective tissue cells does not seem to contribute significantly to the volume increase(3). Earlier histological studies indicated that mucopolysaccharides are present in the ground substance of the connective tissue of the rooster comb; this has been confirmed by the isolation of hyaluronic acid from the comb(4). It has been found that testosterone treatment increases the hexosamine content of the young chicken comb(5), but no similar studies have been reported on the capon comb after hormone treatment. Comparing the hexosamine content of the capon with that of the rooster comb, we found considerably higher values in the latter, while histochemical evidence indicated that acid mucopolysaccharides are present only in the rooster comb(6). The aim of the present study was to investigate the histochemical changes in the capon comb after testosterone treatment and to correlate these changes with the results obtained from determinations of the hexosamine, hydroxyproline and water content of the comb. The other head appendices, *i.e.* the wattles and the earlobes, have also been included in this study. Both are known to react to hormone treatment. However, while the comb and the wattles are similar in structure, the earlobes are made up of a morphologically different tissue. We also extended this investigation to the study of the normal development of the comb in untreated young cockerels.

Material and methods. 18 White Leghorn capons, approximately one year old were castrated at the age of 4 to 6 weeks and treated, with the exception of 4 control animals, with daily doses of 2 mg testosterone propionate ("*Neohombreol*," *Organon*)‡ for periods of 3 to 13 days. For comparison, the data obtained on 10 White Leghorn roosters of the same age are included. The development of the comb was studied in cockerels from the time they were hatched until 15 weeks of age, using a total of 82 animals.

Sampling the comb for a comparative study of its different stadia presents some difficulty due to the peculiar form of this organ and its anisometric volume increase. This is illustrated in Fig. 1, where the gradual increase in the area of the capon comb during hormone treatment is shown. It is obvious that in the course of this process each point of the comb moves at a different speed and in a different direction. By taking transverse slices from the comb at the plane of arrow A (Fig. 1), one can obtain sections which represent analogous parts of the comb in different animals. In studying such sections of the capon comb after different periods of hormone treatment, we found that the changes in the connective tissue start at the base of the comb and proceed upward from the skull toward the spikes(3). Such transverse sections might have obvious advantages for a morphological and a histochemical study. However, because the composition of these sections is not uniform, correlation of the results of chemical analysis

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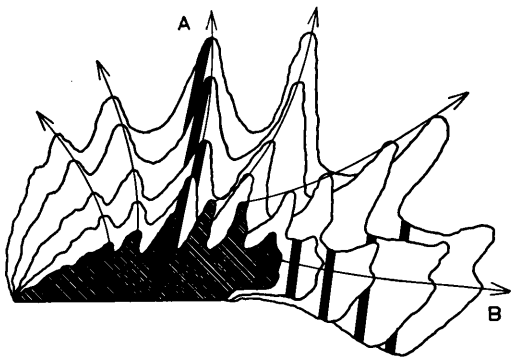

FIG. 1. Schematic representation of increase of comb area in the capon in the course of testosterone treatment (2 mg daily). Based on contact photographs taken every second day during first 8 days of treatment. Heavy black lines indicate location of samples.

with the histological structure is much more difficult. For this latter purpose, we used comb slices cut perpendicularly to arrow B (Fig. 1), which represent a more homogeneous cross section of the process at different time intervals.

The histological and histochemical methods, including the sectioning of the fresh frozen tissue, the periodic acid-Schiff reaction (PAS) and metachromatic staining with toluidine blue, have been described in detail(6). In a few cases the tissue, after fixation in Pfuhl's mixture (sublimate, picric acid, glacial acetic acid), was embedded in paraffin and stained with haematoxylin-phloxin or azocarmin-orange G-aniline blue (AZAN). Hexosamine determinations were carried out according to the modified Elson-Morgan method described previously(6). Comb slices of about 300 mg were used for this purpose except in very young cockerels, where it was necessary to use the whole comb or even pooled samples of several combs. Prior to hydrolysis, the tissue samples were extracted by acetone and dried in a desiccator to constant weight. This dry weight of the defatted tissue has been used to calculate the water content. The method described by Neuman and Logan(7) has been used for the determination of the hydroxyproline content. The data reported on the hexosamine, hydroxyproline and water content represent averages of determinations made on 10 combs of roosters, 4 combs of untreated capons, and 2 combs from each group of treated capons; while in the case of cockerels, the number of combs used varied between 3 and 8. The ratio of hexosamine to hydroxyproline has also been calculated.

Results. Testosterone-treated capons. The average weight of the untreated capon comb was 1.57 g, while the rooster combs measured about 45 g. After 13 days of testosterone treatment of capons, the average comb weight was 21.2 g. A detailed description of the connective tissue of untreated capon comb is given in previous reports(3,6); here we shall mention only that there is no metachromatic ground substance present in the capon comb and that the hexosamine content is rather low (0.53% dry weight). Estimating the hydroxyproline content in the comb of untreated capons, we found an average value of 3.65% dry weight, resulting in a ratio hexosamine: hydroxyproline of 0.15. Studying the changes in the capon comb after testosterone treatment, we found after 3 days that there was already a large amount of metachromatic material present in the intermediate layer, especially in the lower parts of the comb. The gradual development of this mucoid tissue is shown in Fig. 2, 3 and 4, which are taken from different parts of the same transverse section through the middle of the comb. Fig. 2 shows the very first appearance of the metachromasia in the spike; Fig. 3 shows further development in the middle part; while Fig. 4 represents a fully developed mucoid tissue at the base of the comb close to the skull. In comparing sections from the combs of capons after different periods of treatment, 3, 5 and 13 days respectively, we found that after 3 days the fully developed mucoid tissue is restricted to the basal parts, while after 5 days almost the whole section contains this layer. Finally, after 13 days, the intermediate layer throughout the entire comb is transformed into mucoid tissue, occupying all of the space between the reticular layer under the epithelium and the central core. The appearance of the metachromasia is the main occurrence histochemically. We did not find any change in the PAS positivity, which was restricted to the collagen fibers(6).

The wattles show essentially the same

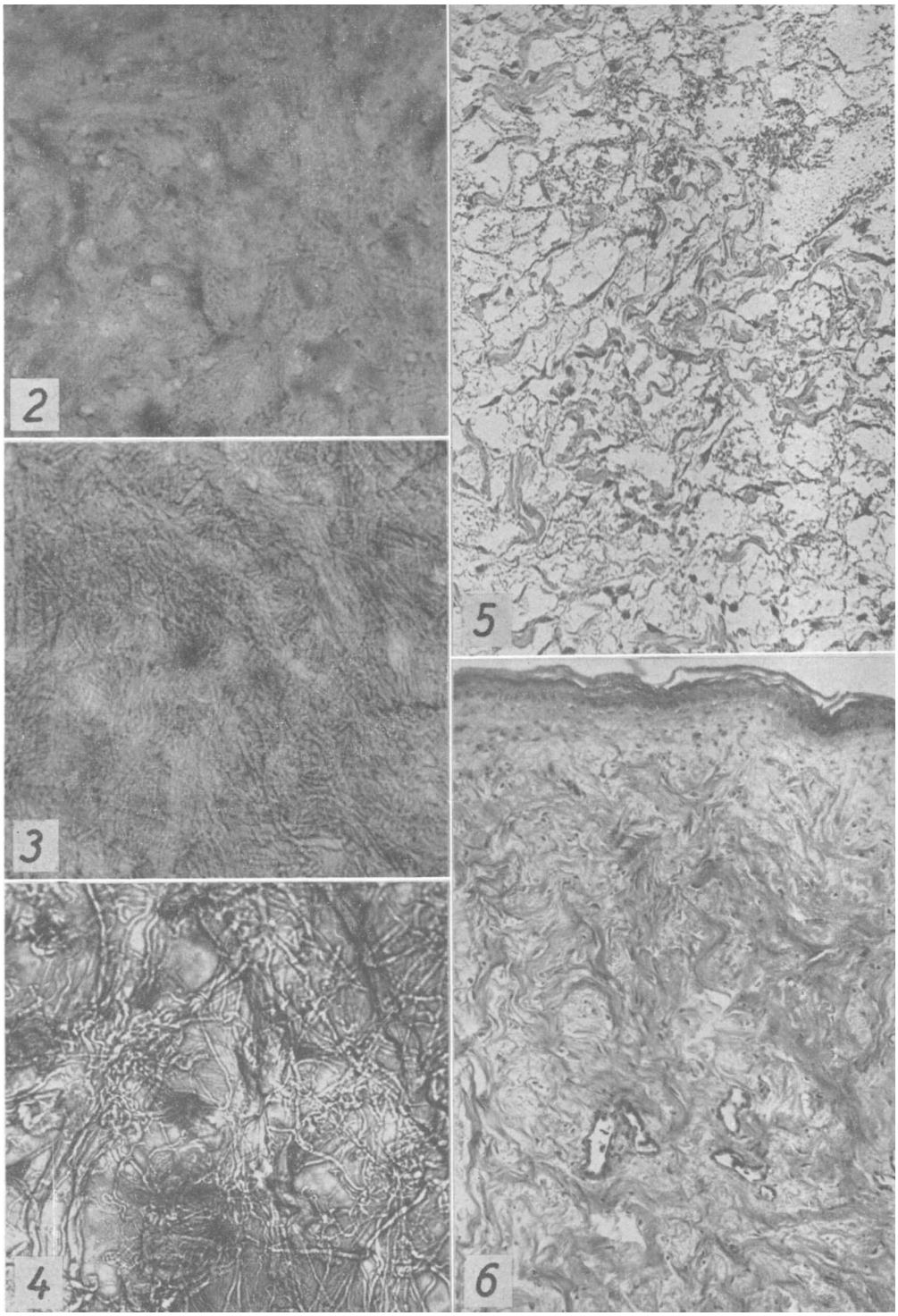

FIG. 2. Capon comb after 3 days of hormone treatment. Frozen section 12.5 μ, fixation in osmic vapor and lead subacetate, stained with toluidine blue (magnification 360×). Mucoid layer in the spike.

FIG. 3. Same section and magnification as in Fig. 2. Mucoid layer in middle part of comb.

FIG. 4. Same section and magnification as in Fig. 2. Mucoid layer in basal part of comb near skull.

FIG. 5. Subcutaneous connective tissue of the wattle of a capon after 3 days of testosterone treatment, showing edema. Fixation: Pfuhl, paraffin section 8 μ, stained with AZAN (magnification 320×).

FIG. 6. Earlobe of untreated one-year-old rooster. Fixation: Pfuhl, paraffin section 6 μ, stained with haematoxylin-phloxin (magnification 180×). Note absence of blood vessels under epithelium and presence of thick, collagen bundles in dermal layer.

structure as the comb, both in the capon and in the rooster, and the reaction to testosterone treatment is very similar. It should be mentioned that the mucoid layer here is narrower than in the comb. Therefore, the mucoid tissue represents a smaller portion of the total structure. There is no preferential localization of the very first metachromatic change in the wattles, as in the basal part of the comb. We noticed another way in which the reaction here seems to differ from that in the comb; *viz.* the edematous appearance of the subcutaneous tissue of the wattle, which was especially pronounced during the first days of hormone treatment. At this time, the wattle increases markedly in thickness, and on cutting through the tissue, fluid escapes from the central part. This fluid showed strong metachromasia when toluidine blue was added *in vitro,* but it was technically impossible to demonstrate this substance in the tissue sections. The spaces between the fibrils of this edematous middle part appear empty in the histological preparation (Fig. 5). Later on this edema tends to disappear; after 13 days of treatment, the subcutaneous tissue of the wattles appears to be similar to the structure in the rooster without any sign of edema.

Histologically, the earlobes of both the capon and the rooster differ from the comb and wattle in many ways. In the subepithelial part of the rooster comb and wattle, the so-called reticular layer, there is a large number of sinusoid blood vessels, showing dilatation upon hormone treatment and giving these organs their characteristic red color. These subepithelial vessels are absent in the earlobes, where there is only a small strip of tissue somewhat similar to the reticular layer of the comb and wattle. While the dermal layer of the comb and wattle of the normal rooster and treated capon contains thin collagen fibrils, the dermal layer of the earlobes consists of thick collagen bundles, homogeneous in appearance. A few small, coiled blood vessels are also present here. (Fig. 6). Compared to the very profound reversible changes of the dermal layer of the comb under hormone treatment, the dermal layer of the earlobes shows fewer alterations. In the capon, the whole dermal layer is narrower and there is no metachromasia present. In the testosterone-treated capon and in the normal rooster, this layer is wider, the collagen bundles have a swollen appearance, and diffuse metachromasia, very faint but still distinct, is present throughout the whole dermis. The only effect of hormone treatment on the earlobe which is similar to that on the wattle is some accumulation of fluid in the subcutaneous tissue, but to a much lesser extent.

The results of chemical analysis of all 3 tissues in the treated capons are summarized in Fig. 7. It is obvious that there is a significant increase in the total hexosamine content of the capon comb during hormone treatment. The same is true for the wattles, but to a lesser degree, while in the earlobes the increase in hexosamine, if at all significant, is very slight. The relative amount of hydroxyproline does not change significantly in the capon comb during hormone treatment; this also seems to apply to the wattles and earlobes. When these values are related to each other, as the hexosamine:hydroxyproline ratio, it is obvious that this ratio tends to increase in all 3 organs during testosterone treatment; these values can be even higher than in the normal rooster.

Normal development of the comb in cockerels. In our experiments, the comb weight increased from 0.013 g at the time of hatching to about 15 g at the age of 15 weeks. The histological structure of the comb in a one-day-old cockerel shows a cellular proliferation of the connective tissue of the dermal layer;

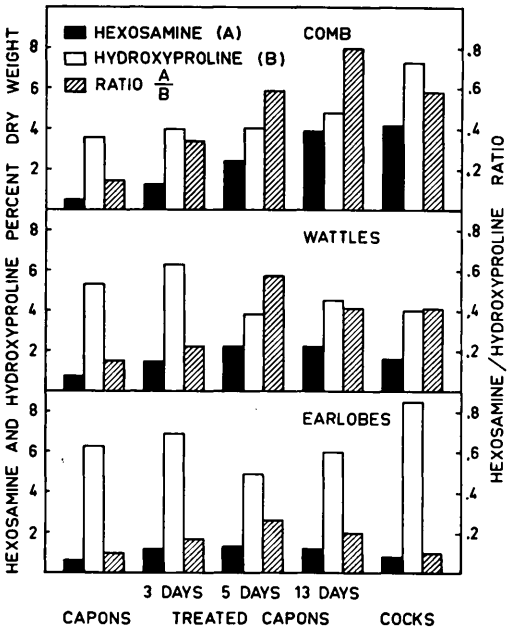

FIG. 7. Hexosamine and hydroxyproline content and hexosamine-hydroxyproline ratio in comb, wattles and earlobes of testosterone-treated capons. For comparison, values from untreated capons and roosters of the same age are included.

the collagen fibrils are already developed, but there is no metachromasia in the ground substance. This picture does not change essentially during the first 3-4 weeks, which seems to cover the normal growth period judging from the frequent mitoses observed both in epithelium and in connective tissue. After this period the accumulation of the metachromatic ground substance begins and is again more pronounced in the basal parts of the comb. Until the age of 15 weeks there is a gradual increase in the amount of mucoid tissue in the comb. During this time mitoses still can be seen in the epithelium, but are seldom observed in the connective tissue. At the end of this period the structure of the normal rooster comb has been developed. Chemical determinations (Fig. 8) show that the relative amount of hexosamine in the comb starts to increase very slowly during the first 4 weeks, but increases more rapidly thereafter, reaching the level of the fullgrown rooster comb in about 15 weeks. The relative amount of hydroxyproline is constant throughout the whole development. The hexosamine-hydroxyproline ratio conceivably

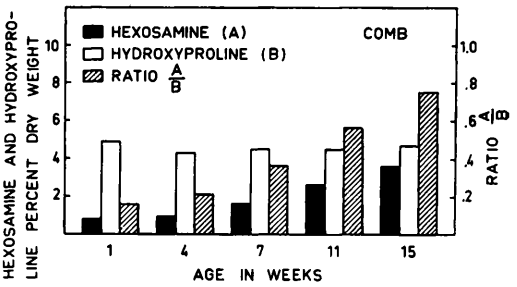

FIG. 8. Hexosamine and hydroxyproline content and hexosamine-hydroxyproline ratio in comb of developing cockerels.

increases during this time.

In Fig. 9 water content data of comb samples are plotted against the corresponding hexosamine values. These figures show that in treated capons the water content is somewhat higher than in normal, untreated roosters and older cockerels.

Discussion. The comparative histological study of the comb development in cockerels and in testosterone-treated capons showed that in both cases the process of "expansion growth" is essentially similar. This process takes 10 to 15 weeks in cockerels; in treated capons it can be completed in about 2 weeks.

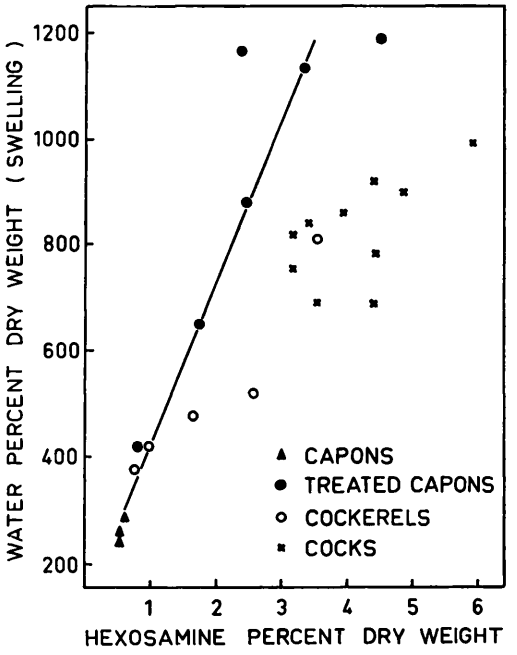

FIG. 9. Water content (expressed in % of fat-free dry wt) and hexosamine content of comb in treated and untreated animals.

In the cockerel, it is preceded by a phase of normal tissue growth which takes place during the first 3-4 weeks after hatching. Mitoses in the connective tissue were found only during the first 4 weeks; after that, during the "expansion growth" period, we rarely found cell division in the mucoid layer. Ludwig and Boas(8) and Schiller, Benditt and Dorfman (5) observed active cell proliferation in the connective tissue of the comb in young cockerels receiving androgen treatment. The animals used were 3-24 days old(8) and 10 days old(5) respectively. A possible explanation for this difference could be that in animals so young the process of "expansion growth" overlaps the normal growth process. Determination of the total hexosamine shows that there is an increase of some hexosamine-containing substance or substances in both treated capons and normally developing cockerels in the course of the "expansion growth." This applies especially to the comb, but also seems to occur in the wattles and possibly in the earlobes. It should be mentioned that our data do not allow any further interpretation of this increase in hexosamine content, but they are in all cases parallel to the metachromasia as found under the conditions of the histochemical test. This altogether might be evaluated as indirect evidence for the supposition that one of the main phenomena in the hormonal response of these tissues is the accumulation of mucopolysaccharides in the ground substance, probably hyaluronic acid. Since the concentration of hydroxyproline in the treated capon remains relatively constant in the course of the "expansion growth," this indicates that there is also an active formation of collagen during the process which is proportional to the increase of the total fat-free dry mass of the comb. Compared to this the increase in hexosamine content is much larger. The same applies to the increase in water content, which is parallel to the increase in hexosamine content, suggesting that these two phenomena are in some way related to each other(2). This is supported indirectly by the previous observation that the water content of the isolated mucoid layer of the rooster comb as well as its hexosamine content are much higher than the values obtained on the total comb(6). Another factor which might partially explain the high water content of these organs is the subcutaneous edema which develops in the course of hormone treatment, especially in the wattles and earlobes. Finally, the vasodilatation will increase the blood content of the tissue and also may contribute to the high water content.

Summary. It has been found that the increase of the metachromasia in the connective tissue of the comb coincides with the increase of the total hexosamine content during the development of this organ in the normal rooster, as well as during the testosterone treatment of the capon. In both cases, there is an increase in the water content, suggesting that the presence of mucopolysaccharides in this tissue and the high water content are related phenomena. The relative hydroxyproline values remain essentially unchanged during this reaction, indicating that increase in collagen content is proportional to total increase of the dry substance. The ratio hexosamine:hydroxyproline thus shows an increase in both cases. A similar increase was also found in the wattles and, to a lesser degree, in the earlobes. The wattles show the same structure and reaction as the comb. In the earlobes there is a very slight increase of the metachromasia in the course of hormone treatment which, together with the slight increase in the hexosamine:hydroxyproline ratio, would suggest that the reaction here is also similar, but much less pronounced. During testosterone treatment a transient edema of the subcutaneous tissue has been found, especially in the wattles and earlobes.

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