INFLUENCE OF TESTOSTERONE ON COMB DEVELOPMENT IN EMBRYONIC AND YOUNG CHICKS

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ABSTRACT

Thomas Manning Holt. INFLUENCE OF TESTOSTERONE ON COMB DEVELOPMENT IN EMBRYONIC AND YOUNG CHICKS. (Under the direction of Irvin E. Lawrence, Jr.) Department of Biology, June, 1968.

Chick embryos of the White Leghorn breed were subjected to testosterone-propionate from the 6-6½ day of incubation (stage 29) through the 20-21 day (stage 46), and from hatching through the 4th, 6th, 10th, and 17th days of age. The influence of testosterone on comb morphogenesis was determined by histological and histochemical analyses of comb organs removed at each developmental stage.

Data accumulated in this study indicate that embryonic comb fibroblasts and adipose cells are sufficiently differentiated to respond to testosterone applications at an earlier stage than normally occurs during comb development. Structural materials such as collagen, reticulum precursors and lipids were observed in testosterone treated combs before controls. Other components, e.g., mucopolysaccharides, however, demonstrated more complex assimilation patterns in response to the hormone.

During normal and hormonally stimulated comb growth the production of mucoidal ground substance and collagen fibers proceeded from the base of the comb upward, while overall organ growth was observed to progress along a posterior to anterior axis.

Although the most striking developmental changes in response to testosterone were observed in the three layers of the comb dermis, some change was also noted in the mucopolysaccharidal development of the outermost epidermal layers.

LOVINGLY DEDICATED TO

MY GRANDMOTHER,

MRS. T. J. HOLT

AND TO

MY WIFE, LINDA

SON, JEFF

AND

DAUGHTER, GWYNN

ACKNOWLEDGEMENT

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INTRODUCTION

The comb organ of the chicken is a developmental system that can be readily subjected to experimental conditions (Hardesty, 1931; Lawrence, 1963, 1964). Through an analysis of the effects of various alterations and substances on growth and differentiation of the comb, one may hope to learn more about the interacting control mechanisms involved in developing organ systems (Locke, 1966).

The problem of comb development has been approached from several aspects. The most often used approach has been the analysis of the effects of hormones, e.g., testosterone, on the definitive comb organ. Initial reports on testosterone were given by Berthold in 1849. Later, Stephenson (1915) and Hardesty (1931) gave more complete analyses of comb growth as influenced by testosterone. In the last two decades, comb research has shifted more towards the histochemical approach, with emphasis being placed on the cellular response (Mancini, et.al., 1960; Szirmai, 1956). The comb has often been used as an assay organ for comparative studies of the effects on development of different drugs and other substances (Levine, 1963; Dorfman and Dorfman, 1962). Other workers have used the techniques of antibody production (Caridroit and Moszkowska, 1948), of antagonistic compounds such as methylcholanthrene (Hertz and Tullner, 1947), and of estrogen applications (Hardesty, 1931; Boas and Ludwig, 1950) in analyzing inhibitory responses in the comb.

Most of the research to date has dealt with the combs of either post-hatched, young, capon, or adult chickens. From the data, it is

established that the comb response to testosterone in older chickens is manifested as an increased growth through hypertrophy and hyperplasia of fibroblasts, accompanied by an increased metabolic activity in these cells (Hardesty, 1931; Mancini, et.al., 1960).

The purpose of the present study was to investigate embryonic comb development under the influence of testosterone-propionate. It was desirable to determine not only the specific developmental stage at which comb cells exhibit a competence to respond, but also the mechanism of the response, i.e., the morphological and/or physiological changes in comb tissue cells. The results of this investigation confirm previous reports of response patterns in comb tissues and reveal that embryonic and adult affects are somewhat similar. The data also show that comb cells are sufficiently differentiated to respond to testosterone applications at an earlier stage than is the case in normal development.

THE REVIEW OF LITERATURE

Comb Development: Single comb development proceeds from an initial appearance, around day seven of incubation (stage 30), of an ecto-mesodermal elevation on the frontonasal process. This elevation, or comb ridge, results from a proliferation of underlying mesenchymal cells (Hardesty, 1931).

Lawrence (1963), using homoplastic grafting techniques, found that presumptive comb tissue cells are determined before the fourth day of development (stage 24) in the New Hampshire Red. An earlier determination (stage 15) was found to occur in the White Leghorn breed (Lawrence, 1964). He also reported that the mesodermal portion of the comb organ is responsible, not only for this determination, but also for the pattern of development exhibited.

By the eighth day of incubation (stage 33), the comb area is delineated from the rest of the head by the appearance of feather germ papillae. These papillae cover all parts of the head excepting a narrow central region extending posteriorly from the beak, and conforming to the comb area, and other limited regions around the eyes and external ear orifices. The anterior margins of the comb area at this stage in development are indistinguishable from beak, however, by the tenth day of incubation (stage 36) a line of demarcation is clearly established by a V-shaped notch, the apex of the V pointing posteriorly with comb tissue lateral to the notch and beak material filling the notch medially (Hardesty, 1931).

The comb area at stage 36 is more extensive than the actual comb ridge, and even at maturity the total comb area is never completely elevated. Small papillae, representing the points and blade of the definitive comb organ, arise at intervals along the length of the elevation. The elevation is very low and narrow, with only minute spaces separating the point papillae. By the twelfth day of incubation (stage 38), the comb elevation with differentiating points and blade, still covers only the anterior one-half of the total comb area and, in cross section, only about one-third the width. The external nares mark the anterior boundary of the comb area with the posterior limits being identified only by skin lacking any feather development (Hardesty, 1931).

Comb Histology: The initial comb elevation consists of a bi-layered epidermis derived from ectoderm, and underlain by loose non-comb mesenchymal material. A detailed description of the embryonic and adult comb organ is given by Hardesty (1931). She describes a two layered epidermis, superficial epitrichal and subjacent mucous layers, that undergo modification into a trilayered structure by the ninth day of incubation (stage 35). Appearance of the third epidermal layer, transitional, is attributed, at least initially, to a proliferation of mucous cells. Continued proliferation of mucous cells, with some contribution from epitrichal cells, results in an intermediate multi-layered transitional region by the thirteenth day of incubation (stage 39). The cells of the transitional layer continue to divide and differentiate, resulting, by the fourteenth day (stage 40), in a peripherally placed horny layer, replacing the epitrichal layer. Cells of the horny layer are thus derived through mitotic activity of mucous

cells, differentiating intermediate and epitrichal layer cells (Hardesty, 1931).

The dermis is more complex in its developmental anatomy. The seven day (stage 31) comb dermis shows only compacted mesenchyme below a clearly defined epidermis. By the eighth day (stage 34), the basal mesenchyme begins to differentiate into elongate spindle shaped cells, mostly fibroblasts, which become aligned horizontally in more or less parallel layers by the tenth day (stage 36). A definitive three layered dermal pattern seen in the adult comb is established by the twelfth day of incubation (stage 38) (Hardesty, 1931).

The first appearance of a fibrous network is at about seven and a half days of development (stage 32) and is responsible for the collagenic pattern that is later observed (Lawrence, unpublished). This fibrous network is brought about by the activation of fibroblast cells of the trilayered comb dermis. Porter and Pappas (1959) have demonstrated the formation of collagen fibrils by fibroblast of the chick embryo back and neck dermis. The peripheral layer of the dermis consists of compacted reticular fibers beneath a distinct basement membrane (Lawrence, 1963). Hardesty (1931) describes the central zone as consisting of prominent collagen fiber bundles, almost all of which are directed in a plane perpendicular to the skull and parallel to the plane of the comb elevation. Occasionally, these bundles bifurcate and fibers extend laterally to enter the intermediate layer. The intermediate layer is composed of loosly scattered fibrils that describe a reticular framework (Lawrence, unpublished). In later stages, this layer is readily identified by reticular and collagenic fibers that lie perpendicular to

the lateral surfaces of the comb and vertical to the core. It is this intermediate layer that responds most strikingly to testosterone. Following testosterone injections, a mucoid ground substance accumulates in the intercellular and interfibrillar spaces of the intermediate layer (Hardesty, 1931; Ludwig and Boas, 1950; Szirmai, 1956; Mancini, et.al. 1960; Doyle, 1961).

In the definitive comb, the width of the peripheral zone is reported as being consistant in all regions of the organ. The intermediate zone is much wider in the basal comb regions than in the points. In the central zone the width is broader basally and tapers towards the tip of each point. The variation is due to large deposits of lipid material in the basal regions (Hardesty, 1931). In the embryonic condition no known report has been made on the lipid content of this central zone.

Hormonal Influence on Development: Hormonal influences on organ differentiation patterns has been recognized for some time, although the mechanism for the effects is not clearly understood. Jellinck (1967) gives data from several sources which point toward the derepression of specific gene loci by certain hormones. This process implies the hormonal control over DNA dependent RNA synthesis and thus the synthesis of new enzymatic proteins. Ecdysone injection into Chironomus larvae (Clever, 1964) and testosterone-propionate injections into immature or castrated rats result in an increase in synthesis and activity of RNA polymerase followed by an increase in protein synthesis (Brewer, 1966). The appearance of newly synthesized enzymes can significantly alter a cell's metabolic pathway, resulting in the formation of new end-

products. If these metabolic products are retained within the cell, then they may modify the intracellular chemical balance previously established and in so doing alter the mode of differentiation either physiologicially or structurally. If the metabolite, on the other hand, is secreted into the intercellular regions, it may affect the microenvironment and initiate interaction between groups of adjacent cells differentiating along dissimilar lines (Spratt, 1964 and Talwar, 1965).

Besides the local type of hormonally controlled cell interaction, a distant type of control occurs when the target cells are glandular. The accumulation of newly formed cellular by-products secreted into the circulatory system by target cells of a differentiating endocrine gland has a reciprocal effect on the gland responsible for the initial hormonal release (Willier, et.al., 1955; Bonner, 1961). This reciprocal effect usually causes a decrease in the amount of hormone being produced by the initiating gland, and/or promotes the increase in secretion of a third hormone from yet another developing endocrine gland (Willier, et.al., 1955).

Although the data presented in support of a theory for hormonal triggering of inactivated genes appear valid for some epithelial organs and muscle tissues, the affect on a predominately connective tissue organ, such as the chicken comb may be different. Talwar (1965) reports that neither topical nor subcutaneous applications of Actinomycin D with testosterone-propionate had an inhibitory effect upon the growth response of the comb organ. Since Actinomycin D is known to block the synthesis of mRNA (Clever, 1964), these data imply a mechanism other

than protein synthesis for the expression of hormonal influence on comb growth. Talwar mentions, however, that the Actinomycin D may have failed to saturate the comb tissues, thus, no blocking effect on mRNA production could be manifested.

The concept of endocrine control over differentiation implies a delicate, yet dynamic, balance between hormones within differentiating organ systems and tissues and the developmental pattern of the organism as a whole.

Hormonal Influences on Comb Growth and Differentiation: An interplay between the differentiating pituitary and gonads, is a major contributing factor in the development of sex structures and characteristics in the chick. Gonadotrophic hormones produced by the developing adenohypophysis tend to increase their effect as gonadal development progresses (Willier, et.al., 1955). Prior to, during, and just after the onset of morphological sex differentiation, Domm (1937) treated chick embryos with a pituitary extract and found that the gonads of both sexes hypertrophied. This hypertrophy did not effect an increase in the production of the sex hormones because no significant increase in comb size was observed. Following pituitary extract injections to newly hatched chicks, however, there was an increase in comb growth which indicates greater responsiveness of the gonadal tissues at this later stage in development (Willier, et.al., 1955). Marlow (1951), injecting frozen extracts of chick pituitaries, extracts of fresh bovine pituitaries, and pure samples of ACTH into capons, found a species specific substance(s) in the chick pituitary gland responsible for an increase in comb growth.

Other hormonal influences in comb formation of the male chick exist between the testes and the thyroid gland. Morris (1951) describes the influence of thyroxin and androgen on comb growth as a synergistic one, based on weights of the comb, gonad, and thyroid gland following various combinations of injections of testosterone-propionate and thiouracil. Caridroit (1942) reports that thyroxin intensifies the metabolism of the receptive tissues of the comb and thus increases their reaction to testosterone. In addition, he states that the comb tissues have a lowered threshold of response to testosterone following pre-treatment with thyroxin. Caridroit (1943) indicates that thyroxin not only enhances the reaction of the comb to androgenic stimulation, but that it also prolongs it.

To summarize the possible hormonal control over male comb differentiation, it seems that the initial, simultaneous, release of hypophyseal thyrotrophic hormone and adenohypopyseal gonadotropic hormone, which occurs on the eleventh day of incubation (stage 37), stimulates the thyroid and testes to secrete thyroxin and androgen, respectively, into the circulatory system (Willier, et.al., 1955). Upon reaching the comb tissues, thyroxin stimulates the comb cells to increase their metabolic rates and androgen (testosterone) initiates a growth response. The net effect is a rapid increase in the overall size of the comb.

Comb Tissue Response to Testosterone: Comb tissue, when exhibiting hypertrophic growth response to androgenic stimulation, is a target organ for testosterone and its many derivatives. It can be utilized, therefore, as a test organ for assaying the nature of various androgens

and other compounds as growth enhancers or inhibitors (Dorfman and Dorfman, 1962).

As has been previously mentioned, the response displayed by the comb (embryonic, post-hatched, or adult) to testosterone concentrations, is primarily the result of a synergistic hormonal interaction based upon the effects of thyroxin and testosterone on the target cells (fibroblasts) within the comb structure.

The thyroid gland becomes histogenetically functional at about the eleventh day of incubation (stage 37), and the testes from the eighth to tenth day (stages 33-36) (Willier, et.al., 1955), consequently, one can assume that some thyroxin and testosterone will be interacting with the comb fibroblasts from this time on in development.

The response to testosterone in immature male chicks is described by Ludwig and Boas (1950) as a proliferation, metaplasia, and an increase in the alkaline phosphatase content of fibroblasts in all layers of the comb dermis, accompanied by an incorporation of an intermediate layer deposition of hyaluronic acid. The dense connective tissue, characteristic of young chick comb, is rapidly converted into a loose one, rich in mucopolysaccharide that is localized in the intermediate dermal layer after applications of testosterone (Mancini, et.al., 1960). Mancini attributes the production of the mucoid ground substance to the fibroblast cells and Szirmai (1957) attempted to identify changes in the mast cells of the comb organ related to ground substance formation but could confirm no alteration in their morphology or number subsequent to the testosterone applications. Szirmai (1965) agrees with Mancini

and proposes that the high water concentrations noticed in combs of testosterone treated chicks be linked to the high degree of mucopolysaccharide formation.

Several different compounds which have been either isolated from or at least tentatively identified in comb tissue following testosterone treatments include: chondroitin sulfuric acid (Mancini, et.al., 1957); hyaluronic acid (Ludwig and Boas, 1950 and Mancini, et.al., 1957); acidic glycosaminoglycans (Doyle, et.al., 1964); hexosamine; and hydroxyproline (Szirmai, 1965).

Doyle (1964) has shown that during regression (i.e., after pretreatment with testosterone for a period followed by discontinuation of hormone) the hyaluronic acid of the comb is rapidly metabolized, the water content drops markedly, and an eventual breakdown of the collagen framework occurs. These data indicate that the growth response initiated, maintained, and prolonged by the stimulatory activity of testosterone is a dynamic one and can be reversed by removal of the testosterone.

METHODS AND MATERIALS

Fertilized eggs of a pure White Leghorn breed were obtained from Castlebury's Poultry Farm, Apex, North Carolina, and held from one to five days at 17 degrees Centigrade before incubation. The eggs were placed in a forced air incubator according to a predetermined schedule for developmental staging. While under incubation, the eggs were exposed to an ambient temperature of 37.5 degrees Centigrade and 85 per cent relative humidity and turned twice daily.

After an elapsed incubation period suitable for embryonic growth to a desired pre-injection stage, the eggs were candled to insure proper development, then opened, using a dental drill and abrasive disc apparatus. An initial opening was cut in the blunt end of the shell to rupture the air space and relieve internal pressure, so that the embryo would drop to a lower and more protected position within the shell (Wenger, 1951). Then, a square hole was cut through the shell and the underlying fibrous membrane removed to expose the embryo. This technique provided a means for selecting a more suitable injection site.

Injections were made using a 25 gauge hypodermic needle attached to a tuberculin syringe graduated in .01 cc units. Due to variences in the embryonic stages being tested, and because there existed a 2-5 day incubation age difference between the times of injection and comb removal, the injection quantities and testosterone concentrations varied as follows:

developmental stage at comb removal	volume injected	testosterone concentration per unit volume
29-32	0.05 cc	2.50 mg
33-34	0.10 cc	5.00 mg
35-46	0.20 cc	10.00 mg

Newly hatched chicks of four, six, 10 and 17 days were given daily topical applications of 5.0 mg of testosterone-propionate dissolved in 0.1 cc of sesame oil. The testosterone treatments began on the day of hatching.

Injection was usually into the yolk sac, though, occasionally into the amnionic cavity, the extraembryonic coelom, or the allantois. After injections of the testosterone sesame seed oil solution (experimentals) or of pure sesame seed oil (controls), the shell opening was covered with an 18 mm circular cover slip and sealed with paraffin. The opening into the air space was also sealed to prevent desiccation and possible contamination by micro-organisms. Aseptic conditions were maintained throughout the period of shell opening, injection, and closing phases. Immediately after sealing, the eggs were returned to the incubator and allowed to continue development for an additional period of from 48 to 128 hours. Most of the embryos were sacrificed after 70 to 80 hours of post-injective development.

After killing an embryo and determining its developmental stage as standardized by Hamburger and Hamilton (1951), the comb organ was immediately excised and placed in an aqueous saturated mercuric chloride

fixative. The fixation period varied from one day to two weeks, with the majority of tissues remaining in the fixative only 24-48 hours. Following fixation, the combs were washed in 70 per cent ethyl alcohol to remove excess mercuric chloride, dehydrated in the standard alcohol series, cleared with xylene, and impregnated for 8-16 hours in paraffin (melting point 56.5 degrees Centigrade). After infiltration, the tissues were imbedded in paraffin and stored at room temperature until sectioned. Combs of each developmental stage were serially sectioned at ranges from five to 11 microns. Table 1 presents a summary of these combs.

Several different staining procedures were employed to provide a broad histochemical picture of comb histogenesis. The Gomori Trichrome stain was used for connective tissue fibers. The Hale Colloidal Iron-PAS method provided information on connective tissue mucins and ground substance. Lillie's Reticulum Technique was employed for reticular fibers and membranous structures. The Alcian Blue 8GX Schedule was also used for connective tissue mucins. The developmental pattern of epithelial tissue mucins was demonstrated by Lillie's Allochrome method. All these staining techniques were taken from Lillie's Histopathologic Technique and Practical Histochemistry. Table 2 shows the number and stages of combs stained with each of the above techniques.

For comparative analyses, graphic drawings were made of testosterone treated and control combs at each developmental stage. Measurements for the reconstructions were recorded for every tenth section. Width measurements were determined with an ocular micrometer for the peripheral, intermediate, and central zones of the comb dermis. This was done so

Table 1 **Legend:** * Hamburger and Hamilton, 1951.
(Normal chick stage series)
T Testosterone treated embryos
C Control embryos
DPH Day post hatch

TABLE 1

SUMMARY OF COMBS STUDIED

Stages*	No. Imbedded		No. Sectioned	
	T	C	T	C
29	16	3	5	3
30	7	6	3	2
31	6	5	2	2
32	18	6	7	3
33	15	4	6	2
34	7	9	2	4
35	17	13	11	9
36	9	3	6	5
37	18	11	7	6
38	12	6	11	8
39	8	5	7	7
40	9	6	5	3
41	5	5	7	7
42	18	16	2	4
43	14	10	7	3
44	21	19	10	9
45	15	9	8	6
46	3	2	1	1
4-DPH	2	2	1	1
6-DPH	2	2	1	1
10-DPH	2	2	1	1
17-DPH	1	1	1	1
Totals	225	145	111	88

that conclusions could be made as to changes in strata configurations under testosterone influence.

Finally, photomicrographs were taken of selected tissue sections that were thought to best represent the histological and/or histochemical changes.

Table 2 Legend:

*	Hamburger and Hamilton, 1951. (Normal chick stage series)
1	Lillie's Histopathologic Technic and Practical Histochemistry, 1965.
2	At least one comb from the indicated series was separated into different portions and stained with each of the methods presented.
T	Testosterone treated embryos
C	Control embryos
DPH	Days post hatch

TABLE 2

STAINING SERIES WITH NUMBERS OF COMBS USED

Stage*	Harris Hematoxylin Method [1]		Hale's Colloidal Iron-PAS [1]		Gomori's Trichrome Stain [1]	
	T	C	T	C	T	C
29	4	2	1[2]	1[2]	1[2]	1[2]
30	1	1	1[2]	1[2]	1[2]	1[2]
31	2	1	1[2]	1[2]	1[2]	1[2]
32	6	2	1[2]	1[2]	1[2]	1[2]
33	3	1	2	1	2	1
34	1	2	1	1	1	1
35	6	3	2	3	2	2
36	1	1	2	2	2	2
37	2	1	2	2	2	2
38	1	1	2	3	5	4
39			2	2	2	2
40			1[2]	1[2]	4	2
41			2	2	2	2
42			1	2	1	2
43			2	2	2	2
44			7	6	2	3[2]
45			5[2]	4[2]	2[2]	2[2]
46			1[2]	1[2]	1[2]	1[2]
4-DPH			1[2]	1[2]	1[2]	1[2]
6-DPH			1[2]	1[2]	1[2]	1[2]
10-DPH			1[2]	1[2]	1[2]	1[2]
17-DPH			1[2]	1[2]	1[2]	1[2]

(Table 2 - continued)

Stage*	Lillie's Reticulum Technique[1]		Alcian Blue 8GX-PAS Schedule[1]		Lillie's Allochrome Method[1]	
	T	C	T	C	T	C
29	1[2]	1[2]	1[2]	1[2]		
30	1[2]	1[2]	1[2]	1[2]		
31	1[2]	1[2]	1[2]	1[2]		
32	1[2]	1[2]	1[2]	1[2]		
33	2	1	2	1	1	1
34	1	1	1	1[2]	1	1
35	1[2]	1[2]	1[2]	1[2]		
36	1[2]	1[2]	1[2]	1[2]		
37	1	1	1	1	1	1
38	1	2[2]	2	1	1	1
39	2	2	1	1	1	1
40	1[2]	1[2]				
41	2	2	1	1		
42	2	2	1	1		
43	2	2	1	1	1	1
44	1	2[2]				
45	2[2]	2[2]	1	1		
46	1[2]	1[2]	1[2]	1[2]	1	1
4-DPH			1[2]	1[2]		
6-DPH			1[2]	1[2]		
10-DPH			1[2]	1[2]		
17-DPH			1[2]	1[2]		

RESULTS

Mucopolysaccharides: Table 3 summarizes the relative concentration of two different mucopolysaccharides observed in the dermal strata of the testosterone treated and control combs. The Hale Colloidal Iron-PAS staining technique, utilized in determing mucoid concentration, is specific for connective tissue mucins, and as such, stains Hale's positive mucin a deep blue, while Hale's negative mucin appears magenta. Due to the consistency with which color intensities were exhibited, a system of numerals was assigned to describe the degrees of staining intensity and thus, indirectly, the concentration of mucoid present. Due to incomplete differentiation of the dermal layers during early developmental stages (29-36), the same intensity numbers were assigned to both peripheral and intermediate layers. In order to plot the intensity changes as they occurred at each developmental stage, three measurements were taken of each comb (testosterone treated and control). Staining intensity measurements were recorded for anterior, median, and posterior comb regions, and the degrees of intensity averaged to give one final value for each comb. This process was performed for areas beneath (medial), and lateral to the comb elevation, and to combs representing each developmental stage.

The colloidal iron-PAS stain (table 3) describes no significant differences between experimental and control combs in the assimilation or maintainence of Hale positive mucoid prior to stage 33. This mucoid was seen rather abundantly as a diffuse substance present in all comb regions of test[1] and control combs from stage 29 to stage 35 with slight

[1] "test" refers to a comb subjected to testosterone

TABLE 3

STAINING INTENSITIES OF DERMAL LAYERS IN THE COMB AREA

WITH HALE'S COLLOIDAL IRON-PAS METHOD

	Hale Positive Mucopolysaccharide												Hale Negative Mucopolysaccharide											
	Lateral						Medial						Lateral						Medial					
Stage*	P		I		C		P		I		C		P		I		C		P		I		C	
	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C
29	3	3	3	3	3	3	3	3	3	3	3	3	-	-	-	-	-	-	-	-	-	-	-	-
30	3	3	3	3	-3	-3	3	3	3	3	-3	-3	-	-	-	-	-	-	-	-	-	-	-	-
31	3	3	3	3	+2	+2	3	3	3	-3	2	2	-	-	-	-	-	-	+0	+0	+0	+0	-	-
32	3	3	3	3	2	2	-3	2	3	-3	-2	2	-	-	-	-	-	-	+0	-	+0	-	-	-
33	-3	3	3	2	2	2	-3	+2	-3	2	2	2	+0	-1	+0	-1	-	-	-	+0	+0	+0	-	-
34	2	2	2	2	-2	2	2	-2	-3	-2	2	+1	+0	1	-1	-1	+0	+0	-1	-1	1	1	+0	+0
35	+2	+1	3	2	2	+1	-3	2	3	2	2	1	1	1	1	1	+0	+0	-1	-1	-1	+0	-	-
36	2	2	3	2	-2	1	3	2	3	2	-2	1	-1	-1	-1	-1	+0	+0	-1	-1	-1	-1	+0	-1
37	+1	1	+2	2	2	+1	-3	+2	2	2	-2	1	1	-1	1	-1	1	-1	1	-1	1	-1	+0	+0

38	-1	-1	1	-1	1	1	2	+1	1	2	+1	1	+1	1	2	+1	+1	1	-2	1	2	+1	1	1
39	-1	-	-	-	1	+1	-2	+0	-1	+0	+1	-2	+1	1	2	-2	+1	1	-2	1	2	+1	1	-1
40	-	-1	-	-	+1	1	+0	-	-	-	-1	1	-2	1	+2	2	1	1	-2	+1	2	2	2	1
41	-	-	+0	-	+1	+1	-1	-1	+0	-	-2	1	-2	+1	+2	2	+1	1	+1	+1	2	+1	1	+1
42	-	-	-	+0	+1	1	+0	+0	+0	-	+1	1	2	2	3	2	-2	+1	+1	1	-3	2	+1	+1
43	-	-	-	-	-2	-	+0	-	+0	-	+1	-1	-3	2	3	2	-2	-2	+2	2	3	2	2	2
44	-	-	-1	+0	-2	-2	-	+0	-1	+0	-2	-1	-3	+2	3	+2	2	-2	-3	2	+4	3	+2	2
45	-	-	-	-	-1	+0	-	-	-	-	-1	+0	-3	+2	+3	-3	3	-3	-3	3	-4	+3	+3	-3
46	-	-	+0	-	-1	-	+0	-	+0	-	+0	-	2	3	2	3	+2	2	+2	3	-3	3	+2	2
4-DPH							2	-1	-	-	2	1							-3	+2	3	+3	+2	2
6-DPH							1	-	2	-	-	-							-3	3	1	+3	3	3
10-DPH							-	-	3	-	-	-							2	3	-	4	3	3
17-DPH							-	-	+4	-	-	-							2	3	-	4	3	+3

Legend:

*	Hamburger and Hamilton, 1951. (Normal chick stage series)
P	Peripheral dermal layer
I	Intermediate dermal layer
C	Central dermal layer
T	Testosterone treated embryos
C	Control embryos
DPH	Day post hatch
-	No mucin stained
1	Slightly stained for mucin
2	Moderately stained for mucin
3	Heavily stained for mucin
4	Very heavily stained for mucin
+ -	Minor variability in stain

intensity decreases occurring during these developmental periods. A rather sharp loss in the concentration of Hale positive mucin occurs between stages 35 and 40, and continues to be gradually degraded as development proceeds to stage 46 (hatching). Although this loss of Hale positive mucin occurs in both testosterone treated and control comb organs, the treated embryonic combs do not lose the mucoid substance as rapidly as do the controls.

Hale positive mucopolysaccharide is seen in test and control peripheral layers in equal concentrations prior to stage 33. From stages 33 to 40 the concentration of this mucin undergoes a noticeable decline in the control comb, while in the test the loss is only slight. No Hale positive mucin is observed in the control peripheral layer by hatching, and only trace amounts are seen in the comparable layer of test. After the fourth day of post embryonic development all traces of the Hale positive mucin have disappeared from the peripheral layer of testosterone treated combs.

The embryonic developmental pattern demonstrated by Hale positive mucin in the intermediate layers of test and control combs is nearly identical to that seen in the peripheral layers. The concentration of Hale positive mucin is the same for tests and controls up to stage 32, with a subsequent loss of this mucin occurring more slowly in test than control combs. In the control intermediate layer the presence of Hale positive mucin is not observed after stage 39, whereas, in the test there is some indication of this mucin up to hatching. A sudden and rapid increase occurs in the assimilation of this mucopolysaccharide by the fourth day of post embryonic development. This accumulation of Hale

positive mucin continues at an accelerated rate up to and beyond the 17th day of post hatched development.

Initially, the central layers of test and control combs give indications of less concentrated Hale positive mucin than the other dermal strata. By about stage 39-40, however, the central layer of both tests and controls demonstrates a higher concentration of this mucin than either of the other layers. This does not represent an increase in concentration of Hale positive mucin by this layer, but rather its slower degradation of the mucin in relation to other layers. The test central layer seems to retain slightly more of the Hale positive mucin than does the same layer in controls. By stage 46 the control central layer gives no indication of Hale positive mucin and the corresponding test layer demonstrates only slight amounts of this mucopolysaccharide. No Hale positive mucin is observed in either test or control central layers from hatching up to at least 17 days of age.

Table 3 also illustrates the appearance, at stage 31, of a mucopolysaccharide staining negatively for the colloidal iron and positively for the periodic acid Schiff portion of Hale's stain. This mucin is designated as a Hale negative mucopolysaccharide.

The first region demonstrating the presence of the Hale negative mucin is the undifferentiated peripheral and intermediate layers of the medial comb area. By stage 33 the lateral comb area also shows the presence of this mucopolysaccharide in the peripheral and intermediate layers. From stage 33 to stage 37 only slight variations occur in the concentration of Hale negative mucin in any test or control dermal layer. There occurs a rapid assimilation of this mucin, however, in all layers

of test and control combs from stage 37-38 (Plate I, Fig. A, B, C, and D). This increase continues steeply, approaching a maximum concentration between stages 44 and 45. The mucoid increase up to stage 45 is apparently not significantly different in the central layers of test and control combs. While a very sharp assimilation difference occurs between test and control intermediate layers, the peripheral layer of the treated comb accumulates only slightly more mucoid than its comparable control layer. A rather sharp decrease occurs in the concentration of Hale negative mucin in the intermediate layer of testosterone treated combs after hatching. Any Hale negative mucin localized in this layer of test combs is completely catabolized by the tenth day of post embryonic growth.

Only slight differences are observed in total mucoid development between lateral and medial comb areas. The Hale positive degradation appears to be more retarded medially in the peripheral layer of both test and control combs. The intermediate and central layers of test combs also appear to be more retarded medially in the degradation of Hale positive mucin. The Hale negative assimilation is detected several stages earlier in the medial comb area than in the lateral areas (31 vs 33), and accumulates in greater quantities in the medial intermediate layer than in its lateral counterpart.

Table 4 analyses the mucopolysaccharide content of testosterone treated and control combs as stained by the Mowry Alcian Blue periodic acid Schiff technique. This staining procedure is positive for acidic mucins, rendering them a deep blue green, but does not demonstrate the metachromatic staining properties of comb tissue mucins as does the

Table 4 Legend:

*	Hamburger and Hamilton, 1951. (Normal chick stage series)
T	Testosterone treated embryos
P	Peripheral dermal layer
I	Intermediate dermal layer
C	Central dermal layer
DPH	Days post hatch

TABLE 4

STAINING INTENSITIES OF DERMAL LAYERS IN THE COMB AREA

WITH ALCIAN BLUE 8GX-PAS METHOD

Stage*	Test 1			Control		
	P	I	C	P	I	C
29	-	-	-	-	-	-
30	-	-	-	-	-	-
31	-	-	-	-	-	-
32	-	-	-	-	-	-
33	+	+	-	+	+	-
34	+	+	-	+	+	-
35	+	+	-	+	+	-
36	+	+	+	+	+	-
37	++	++	+	+	+	-
38	++	++	+	++	+	-
39	++	++	+	++	+	+
40	++	++	+	++	++	+
41	++	+++	+	++	++	+
42	++	+++	+	++	++	+
43	++	+++	+	++	++	+
44	+++	++++	++	++	+++	+
45	++	++++	+	++	+++	+
46	++	+++	+	++	+++	+

colloidal iron-PAS technique.

Traces of the Alcian Blue positive staining mucopolysaccharide are first seen at stage 33 in the undifferentiated peripheral and intermediate dermal layers of test and control combs. Assimilation continues until the mucoid exists as a discernable band lying subjacent to the epidermis at stage 35. The peripheral layer demonstrates a noticeable increase in the concentration of Alcian positive staining mucoid between stages 36 and 37. A similar but belated increase is observed in the peripheral layer of control combs between stages 37 and 38. Both test and control peripheral layers reach their maximum concentrations of Alcian staining mucin between stages 44 and 45 (Plate II, Fig. A and B).

A simultaneous appearance of Alcian positive mucin is seen in the intermediate layer of test and control combs at stage 33, and corresponds exactly to the initial appearance of the mucin in peripheral layers of the same combs. An increase in the concentration of this mucin is seen in the intermediate layer of test combs at stage 37, while a similar increase is not observed in control combs until later (stage 40). Further increases in the Alcian positive mucoid concentration in tests and controls result in a maximum concentration of the mucin in this layer by stages 44-45 (Plate II, Fig. A and B).

The central layers of test and control combs show slight increases in mucoid content over the level attained by stage 39. The central layer of testosterone treated combs, however, gives a positive test for mucin earlier in its development than does the control central layer. There is also some indication that the central layer attains its highest degree of mucin at stages 44-45.

In both test and control combs, there is a slight decrease in the amount of Alcian Blue positive staining mucopolysaccharide from its greatest concentration around stage 44 to hatching (Plate II, Fig. A, B, C, and D).

Fibrous Network: The Lillie reticulum technique utilized in determining comb reticulum pattern employed a silver precipitate onto the reticular fibers. The conditions under which optimal staining would occur were very restrictive, and dependent upon the amount of light and exposure time to the silver solution. Ideally, the reticular network should stain black, while collagen stains in lighter shades of grey, however, as a result of the variability in the staining technique the tissues were occasionally over, or under, stained and reticular fibers were thus difficult to ascertain from collagen.

The establishment of a definitive type fibrous reticulum in the peripheral and intermediate layers by about stage 38, is preceded by the appearance of an extremely fine reticular network in the lateral comb area by about stage 32. There is no discernable difference between reticular fiber density or pattern in test and control combs at this stage. There is possibly, however, a darker staining reaction by the fibroblasts of the test comb to the silver staining technique used. The stage 32 fibroblast of undifferentiated peripheral and intermediate layers in test and control combs seems to exhibit a granular appearance, with few small fibril units attached in a more or less perpendicular fashion to the basement membrane. By stage 35, this network has extended deeper into the peripheral layer and assumes the appearance of a true reticulum. Also by this stage the test comb peripheral layer appears to have a

greater abundance of reticular fibers than the control. There is a corresponding increase in the intensity of fibers in test and control peripheral layers around stage 37. An additional increase at stage 44 results in the pattern observed at hatching. Throughout all the later developmental stages (37-46), the reticulum increase in test peripheral layers is more abundant than that in the controls (Plate III, Fig. E and F). It was difficult to ascertain any reticular pattern in the intermediate layer during later development because of masking by the positively stained collagen fibers also found in this layer.

Table 5 presents an analysis of collagen fibril density as seen in dermal strata of developing treated and control combs. The Gomori Trichrome staining method used here stains collagen fibrils an intense green. The first sign of collagen development in test combs occurs in the central layer of the medial comb area by stage 36. The intermediate layer of the test comb demonstrates the presence of collagen by stage 37, and along with the central layer increases significantly in its fiber concentration by stage 38 (Plate III, Fig. B). The first sign of any collagen in the peripheral layer is observed by stage 39. A second increase in collagen intensity occurs between stages 38 and 39 in peripheral and intermediate layers of test combs (Plate III, Fig. B and D). A final increase is manifested in all three dermal layers between stages 43 and 45.

The collagen development in control combs occurs somewhat later in development than that seen in testosterone treated combs, for the first appearance of collagen is noticed in stage 37. This initial collagen is found in the intermediate layer of the medial comb area. By stage 38,

Table 5 Legend:

*	Hamburger and Hamilton, 1951. (Normal chick stage series)
P	Peripheral dermal layer
I	Intermediate dermal layer
C	Central dermal layer
-	No collagen stained
1	Slightly stained for collagen
2	Moderately stained for collagen
3	Heavily stained for collagen
4	Very heavily stained for collagen
+ -	Minor variability in stain
±	Testosterone treated embryos

TABLE 5

ANALYSIS OF COLLAGEN FIBRIL DENSITY

WITH GOMORI'S TRIPLE STAIN

Stage*	Test ‡			Control		
	P	I	C	P	I	C
29	-	-	-	-	-	-
30	-	-	-	-	-	-
31	-	-	-	-	-	-
32	-	-	-	-	-	-
33	-	-	-	-	-	-
34	-	-	-	-	-	-
35	-	-	-	-	-	-
36	-	-	1	-	-	-
37	-	1	+1	-	1	-
38	-	+2	2	-	2	1
39	+1	3	2	-	2	+1
40	2	+3	2	1	+2	2
41	+2	+3	2	+1	+2	2
42	3	+3	2	2	+2	2
43	+3	+3	+2	+2	3	+2
44	4	4	+2	3	3	+2
45	4	4	3	+3	+3	+2
46	4	4	+3	4	4	3

(Plate III, Fig. A), collagenic fibers are seen in the central as well as intermediate layer, yet it is stage 40 before any collagen is registered in the peripheral layer. As in test combs, there is a final collagen increase in all three dermal layers following stage 43. At hatching, the collagen content of test combs is still greater in all three dermal layers than that observed in controls.

Although there were several intraorgan changes in the density of collagen fibers during development, the embryonic intermediate layer of test and control combs always manifested the most concentrated collagen region. The central layer demonstrated the next most concentrated region, and the peripheral layer the least.

Adipose: Adipose tissue found in the central comb dermis responds to testosterone with signs of hyperplasia and hypertrophy. Plate IV, Fig. A and B, shows that the first indication of hypertrophy occurs by stage 38. From stage 38 to hatching, (Plate IV, Fig. A, B, C, D, E, and F; Plate II, Fig. A, B, C, and D), there is a progressive increase in the numbers and sizes of fat cells present in the central layer of testosterone treated combs. This increase occurs both in medial and lateral comb areas.

The heaviest concentration of lipid in test and control combs lies just medial and basal to the junction between the comb elevation and the lateral comb areas. The lipid cells are first seen (stage 35) as small isolated cells or groups of cells, and by stage 38 as closely related packets of cells of the "mulberry" type (Ludwig and Boas, 1950).

Testosterone initiates a similar response in the isolated and clustered lipid cells. No adipose tissue is seen in the intermediate

or peripheral layers of either test or control combs. In the test combs, however, the lipid cells extend further up into the core than in the controls, and are also located closer to the junction between intermediate and central layers.

Organ Differences: Embryonic comb tissues exhibit similar size increases in response to testosterone as observed with older birds. The earliest size difference observed between testosterone treated and control combs is seen at stage 33. In this stage, the test central layer appears slightly less dense than in the control. Plate V, Fig. A, B, C, and D, shows the broadened test comb base, and overall height difference in the test and control combs as seen by stages 35 and 36. The height is measured from the center of the ethmoidal internasal septum to the apex of the comb ridge.

Once the comb elevation has been clearly established (around stage 35-36), the developmental patterns of mucin assimilation and adipose deposition follow a posterior to anterior axial gradient. The anterior, median, and posterior regions of the central layer in test combs from stages 36-37 broaden out and assume a less dense nature than the central layers of the controls. By stage 38, the anterior central layer is more dense with collagen fibers, yet actually incorporates a smaller volume than the corresponding control layer. This same pattern continues through stage 39 (Plate V, Fig. E and F). By stage 40, the testosterone treated comb again expresses a greater volume in all regions of the central layer. Plate VI, Fig. A, B, C, D, E, and F, compares in anterior to posterior sequence testosterone treated and control combs sectioned at stage 40.

The first measurable indication of a change in the width of the dermal layers in response to testosterone occurs by stage 37 (Table 6), and by stage 39 is clearly defined.

A definitive pattern of response to testosterone is reached by stage 44, where the test comb is much wider and taller, and where the peripheral, intermediate, and central dermal layers are much broader than in the controls.

Plates VII and VIII present graphic reconstructions showing overall size increases between test and control combs at stages 38, 40, and 42.

Plate IX summarizes, in graphic form, the influences of testosterone observed on several intraorgan components of the differentiating comb dermis.

Epidermis: Staining with Lillie's Allochrome Method for epithelial mucins indicated a response by the epidermis to testosterone. There appears to be some difference in the mucopolysaccharide composition between test and control comb epidermis by stage 35. The test outer layers (epitrichal) of the epidermis stains dark red at stage 35, while the control remains pink. There is another change by stage 38 resulting in a blue horny layer in the control, while the test horny layer stains light green. At this stage, as well as, throughout development the mucous layers in both test and control combs stain blue green. By stage 43 the control horny layer appears blue green while the test remains light green. At hatching, the control epidermis is identical to the test, both of which appear light green in the horny layer, blue in the transitional layer, and light green in the mucous layer. There is no change in staining between the testosterone treated or control epidermal layers of newly hatched chicks nor of four day post hatched chicks.

TABLE 6

COMPARISON OF THE COMB DERMAL LAYER WIDTHS

Stage*	Peripheral		Intermediate		Central	
	T	C	T	C	T	C
38[1]	68.00[2]	51.00	121.17	73.91	358.67	342.99
39	81.80	68.47	134.50	78.40	465.80	397.80
40	74.90	68.17	137.49	78.17	497.82	449.71
41	107.10	83.70	146.33	100.98	526.59	459.00
42	105.40	81.00	153.00	102.00	510.00	462.00
43	102.85	85.00	170.00	102.00	544.00	477.49
44	123.68	85.00	166.26	107.44	680.00	520.20
45	142.68	97.44	179.00	113.32	800.53	625.00
46	136.00	119.00	221.55	119.00	1450.00	1020.00

Legend:
- * Hamburger and Hamilton, 1951. (Normal chick stage series)
- T Testosterone treated embryos
- C Control embryos
- 1 Earlier stages were insufficiently differentiated for measurements
- 2 All measurements are expressed in microns

DISCUSSION

The hypertrophic responses to testosterone-propionate by adult combs are, at the tissue level, intracellular and extracellular and reflect a total organ volume increase. Responses are most obvious in the intermediate layer of the dermis and are expressed as increase in activity of fibroblasts (Hardesty, 1931; Ludwig and Boas, 1950; Szirmai, 1956; Mancini, et.al., 1960). These fibroblasts secrete a muocpoly-saccharide ground substance consisting mainly of hyaluronic acid (Ludwig and Boas, 1950), and chondroitin sulfuric acid (Mancini, et.al., 1960). The fibroblast response also involves the production of collagen fibers from materials (e.g., hydroxyproline) at the cell surface (Porter and Pappas, 1950). Hydroxyproline has been observed to increase proportionately to the increase of total fat-free dry mass of the comb while the proportional increase in hexosamine is much larger (Mancini, et.al., 1960). Mancini also reports a large increase in the water content and correlates the concentrations of hexosamine and water to each other, proposing that the two phenomena are directly related.

The large volume increase in the central layer of embryonic combs treated with testosterone in the present study is possibly due to intercellular water accumulation, since the intercellular mucoidal concentration of this layer does not significantly increase. Except for apparent changes in the mucoidal ground substance, as indicated by the staining techniques used, the results of the present study show responses to testosterone as being similar to those already reported for older comb organs.

Hyperplasia and hypertrophy occur in embryonic comb development in response to testosterone-propionate. Hypertrophy, however, is more striking. There was apparent hyperplasia of fibroblasts in peripheral and central dermal layers and obvious hyperplasia of adipose tissue cells in the central layer. Cellular hypertrophy was seen in adipose tissue cells, but never in fibroblast cells. The greatest hypertrophic responses occur in cells of the central layer beneath the junction of the lateral and elevated comb areas. Extracellular hypertrophy, involving mucopolysaccharide secretion and collagen fiber assimilation, was reflected in a noticeable size increase in the total comb organ.

According to the type of staining reaction produced, the mucopolysaccharides observed were catagorized into two groups: Hale positive and Hale negative. Hale positive mucin proved to be equally concentrated in both test and control combs prior to stage 31, and apparently not influenced in its assimilation rate up to that stage by testosterone. After stage 31 there is a gradual degradation of the Hale positive mucin up to hatching which is seemingly retarded in the presence of testosterone. From hatching on to at least the 17th day of age there is a cumulative, localized (intermediate layer) increase in the concentration of this mucin in response to testosterone. The Alcian Blue staining technique reveals the presence of a mucoidal substance which corresponds to the pattern seen in the development of Hale negative mucoid. A correlation of initial appearance of Alcian positive and Hale negative mucins by stage 33, increasing to a maximum by stage 44, and subsequently decreasing from stage 44 to 46, justifies considering these as being the same. There is no evidence in the present study to

indicate that testosterone causes an earlier synthesis of the reticular network, first seen in peripheral comb layers by stage 32. Some intracellular response may be occurring however, for during initial reticulum formation stages there occurs a darker agyrophilic staining by the reticulocytes in test combs. By later stages (35-46) the density of the reticulum is noticeably influenced by the hormone.

In embryonic comb tissue the intermediate layer always manifests a greater concentration of collagen fibers and mucoidal material. Since there are relatively few fibroblasts in this layer, the greater number of fibers and intercellular ground matrix produced indicate a higher metabolic activity level for these cells. The next most concentrated accumulation of collagen fibers is in the central layer. In adult combs, this layer is the most collagen dense. Apparently, there is a long term accumulation of collagen in the central layer, accompanied by a reduced collagen synthesis by fibroblasts of the intermediate layer. This change in fiber concentration may be the result of altered nutrient supply, i.e., change in blood vascular pattern. The peripheral layer always demonstrates the lowest concentration of collagen fibers both embryonically and in adult comb organs.

The data accumulated in this study support the conclusions of other investigators that the fibroblast of the intermediate layer is an important target cell for testosterone stimulation (Ludwig and Boas, 1950; Szirmai, 1956; Mancini, et.al., 1960). It appears that the fibroblasts of peripheral and central layers in embryonic comb, also respond. There is probably a change in competence, or maturation, of the fibroblasts between the earlier stages of reticular fiber synthesis

and the later appearance of collagen fibers. The adipose cells of the central layer seem to be other target cells for testosterone in the embryonic comb organ, which upon stimulation accumulate more lipid material. This hypertrophy of adipose cells, along with the agyrophilic reaction of the peripheral reticulocytes, provide two examples of intracellular response to testosterone.

Androgenic control over embryonic comb differentiation is primarily one of stimulation of metabolic activity of target cells which result in an increased synthesis or storage of cellular products; lipoidal, fibrous or mucoidal. In support of this, Ludwig and Boas (1950) have found that increased cytoplasmic basophilia occurs in almost all cells of young and actively growing tissues in response to endocrine stimulation. This basophilia is a reflection of the amount of RNA present and can be correlated with metabolic activity and protein synthesis by these cells.

In order for the cumulative changes discussed above to be realized, the cells must be competent to respond to testosterone stimulation. The structural materials (i.e. collagen, reticulum precursors, and lipids) demonstrated in testosterone treated combs before control combs show that the cells are competent to respond even though the hormone may not be present. Although collagen is not normally observed until stage 37, testosterone applications reveal a competence by stage 36. Ordinarily, adipose tissue cells hypertrophy around stage 45, but when treated with testosterone, show a competence to respond as early as stage 38. There may even be an earlier capacity to form reticular fibers as seen by the appearance of possible precursor material in testosterone stimulated

reticulocytes as stage 32. A competence to produce reticular fibers earlier than stage 32, however, was not revealed.

The problem of cell competence regarding the synthesis of Hale positive and negative mucins is more difficult to interpret. Testosterone does not reveal a difference in the embryonic competence level for synthesis of Hale positive mucin, though, a capacity for its continued synthesis is disclosed. The renewed production of Hale positive mucin by intermediate layer fibroblast of post hatched chicks in response to testosterone, indicates that a new level of differentiation (or maturation) is reached by these cells shortly after the completion of embryonic development. The competence level for synthesis of Hale negative mucin was not shown to be any different in test or control combs. After the competence stage was reached, however, the testosterone treated fibroblasts did show a capacity up to stage 44-45 to produce more of the Hale negative mucoid than the untreated fibroblasts. The testosterone treated fibroblasts of the peripheral layer showed a decreased capacity to produce Hale negative mucin, the fibroblast of the intermediate layer lost all ability to synthesize the mucin, and those of the central layer seemed to be unchanged in their ability to produce this mucin.

The intercellular spaces between fibroblasts of the central layer are enlarged through water accumulation. This passive increase in the amount of water may be the net result of a decreased synthesis of mucin by these cells. The fibroblasts of the intermediate and peripheral layers, on-the-other-hand, react with an increased synthesis of mucoidal and reticular materials. This overlapping capacity to respond is seen

only in embryonic comb tissues. Possibly the complexity is due to a less definitive differentiation or to an expression of some pleuropotency still remaining in these embryonic cells.

The effect of testosterone upon differentiation as revealed through measurements of the three dermal strata is noticeable as early as stage 38. Differentiation of the layers was hardly complete at this stage, so that some error may have been incurred when determining width measurements. Consequently, the greater differences recorded in test and control intermediate layers at stage 38 may not be reliable. After stage 38, the level of differentiation of this layer made more reliable measurements possible, and progressively greater width increases in tests than in controls are shown. As development continues beyond stage 38, the central layer manifests the greatest width development of any layer in tests as compared to controls. The tremendous size differences between test and control central layers show that this layer is more responsive to testosterone during embryonic development, while in older comb organs there is no outstanding growth response to the hormone. The slight increase measured in the peripheral layer of test combs over that seen in controls indicates a minimal response by this layer during embryonic growth, this is also the case for older combs.

Apparently, there exists a change in the level of response to testosterone by the three embryonic comb strata as post embryonic development proceeds, the central layer decreasing while the intermediate layer shows an increase. The peripheral layer maintains a steady state in its reactivity throughout development.

Growth proceeds more rapidly from the base upward and along a posterior to anterior gradient in testosterone stimulated comb organs. The anterior regions of test and control combs of later stages (40-46) illustrated only slight developmental differences, whereas more medial regions showed moderate response differences, and posteriorly very pronounced changes were observed. A likely reason for this gradential pattern of development may be related to an increased comb area produced posteriorly when the comb ridge was first elevated. The affect would be to increase the mesoderm and thus directly increase the surfact area of the future central layer and would account for more fibroblasts capable of responding to testosterone in this region as opposed to more anterior ones.

There is a competence by the differentiating squamous cells of the outer epidermal layers (epitrichal early in development, and horny during later stages) to respond to testosterone. The first response is in the form of a mucoidal change by stage 35. Still another mucoidal response to the hormone is seen by stage 38 in the form of a color change in these same squamous cells. Beyond stage 38, the testosterone treated cells show no further change in mucoid production over that of controls. This would seem to indicate that the cells of the differentiating horny layer reach their definitive state by stage 38 and no longer respond readily to testosterone stimulation. There is some correlation between the mucoid pattern observed here and the formation of a definitive epidermis by stage 40 (Hardesty, 1931). The stain may possibly indicate the presence of a precursor material that is used in cornification.

SUMMARY

The effect of testosterone-propionate upon the developing comb organ of the White Leghorn breed was studied in embryonic stages 29-46 and post embryonic development of four, six, 10 and 17 days.

In comb differentiation, the production of mucoidal material and collagen fibers proceeds from the base of the comb upward, and overall growth progresses along a posterior to anterior axis.

Based on the data of histological and histochemical analysis of comb development under the influence of testosterone—propionate, the following conclusions were drawn:

1. The first dermal response occurs between stages 32-33 (7½-8 days incubation) and results in a more loose arrangement of fibroblast in the central layer, and in more darkly staining reticulocytes of the peripheral layer.
2. There is a prolonged embryonic synthesis of a mucopolysaccharide which stains positive with the Hale Colloidal Iron-PAS technique, followed by a rapid assimilation of this mucopolysaccharide after hatching.
3. Embryonic comb fibroblasts produce greater quantities of a mucopolysaccharide which stains negative for the Hale Colloidal Iron-PAS technique, whereas, post embryonic fibroblast produce less of this mucin.
4. Hyperplasia and hypertrophy occurs in adipose cells of the central layer.
5. The adipose cells are competent to accumulate lipid material

by stage 38.

6. The fibroblast are competent to produce collagen fibers by stage 36.
7. There is some change in mucopolysaccharide development of the outermost epidermal layers.

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PLATE I

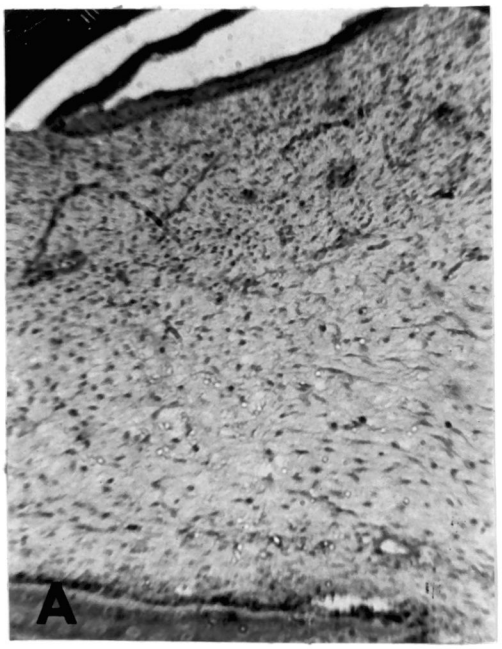
All figures in Plate I are of comb tissue sectioned at 10 microns and stained with Hale Colloidal Iron-PAS technique.

Fig. A. Control comb of a 12-day (stage 38) chick embryo. The cross section was taken near the middle of the comb ridge, and the area represented is at the base of the comb elevation. Magnification: X 440.

Fig. B. Experimental comb of a 12-day (stage 38) chick embryo. The cross section was taken near the middle of the comb ridge, and the area represented is at the base of the comb elevation. Magnification: X 440.

Fig. C. Control comb of a 13-day (stage 39) chick embryo. The cross section was taken near the middle of the comb ridge, and the area represented is at the base of the comb elevation. Magnification: X 440.

Fig. D. Experimental comb of a 13-day (stage 39) chick embryo. The cross section was taken near the middle of the comb ridge, and the area represented is at the base of the comb elevation. Magnification: X 440.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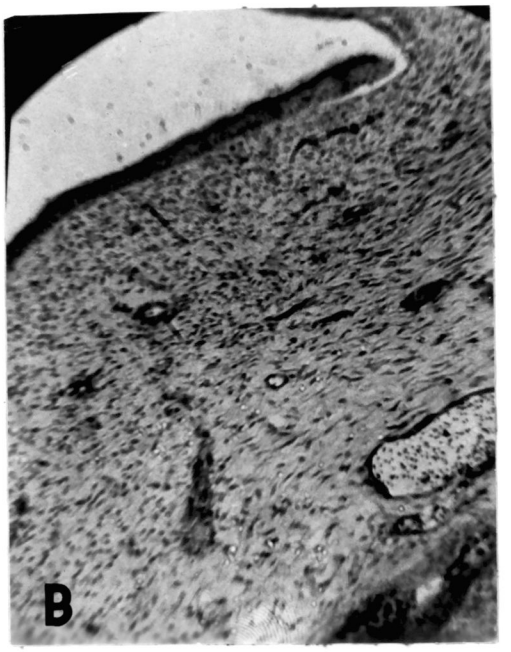
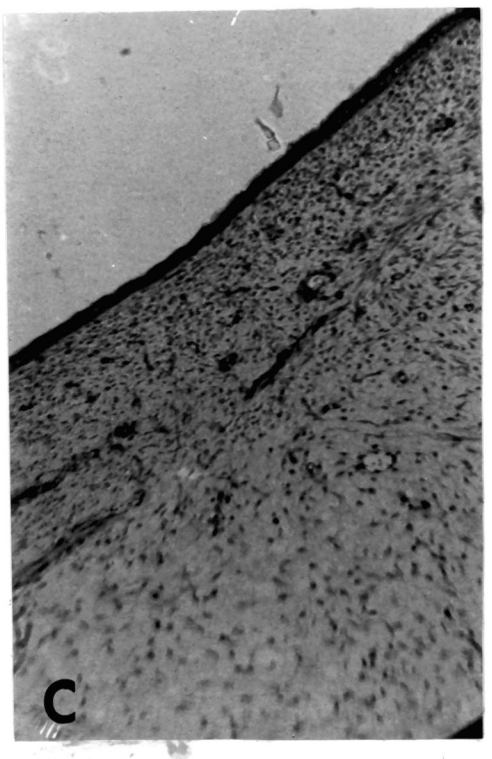

B


C

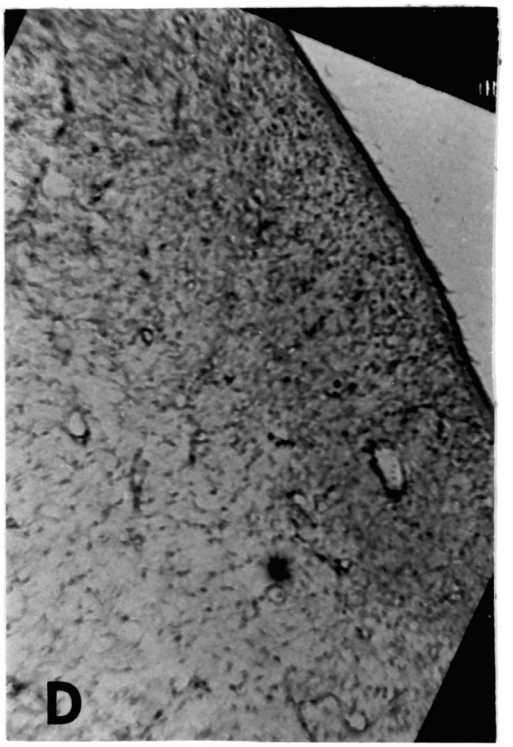

D

PLATE II

All figures in Plate II are of comb tissue sectioned at 10 microns and stained with the Alcian Blue 8GX schedule. The regions represented lie medial and basal to the junction between the comb elevation and the lateral comb areas.

Fig. A. Control comb of an 18-day (stage 44) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 440.

Fig. B. Experimental comb of an 18-day (stage 44) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 440.

Fig. C. Control comb of a newly hatched chick (stage 46). The cross section was taken in the posterior region of the comb ridge. Magnification: X 440.

Fig. D. Experimental comb of a newly hatched chick (stage 46). The cross section was taken in the posterior region of the comb ridge. Magnification: X 440.

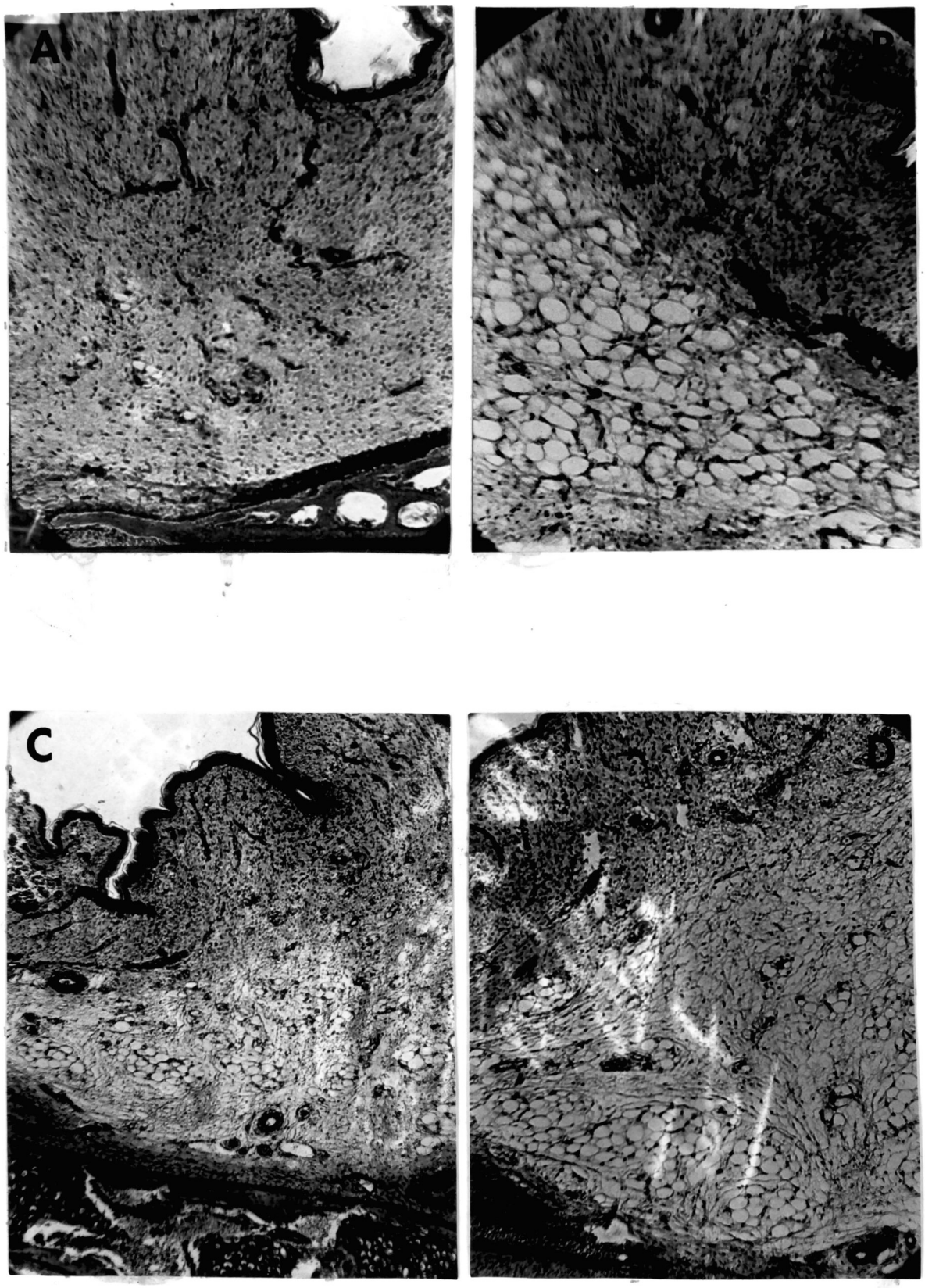

A
B
C
D

PLATE III

Figures A, B, C, and D are of comb tissue sectioned at 10 microns and stained with Gomori's Trichrome Stain.

Figures E and F are of comb tissue sectioned at 7 microns and stained with Lillie's Reticulum Technique.

Fig. A. Control comb of a 12-day (stage 38) chick embryo. The cross section was taken near the middle of the comb ridge, and the area represented is at the base of the of the comb elevation. Magnification: X 440.

Fig. B. Experimental comb of a 12-day (stage 38) chick embryo. The cross section was taken near the middle of the comb ridge, and the area represented is at the base of the comb elevation. Magnification: X 440.

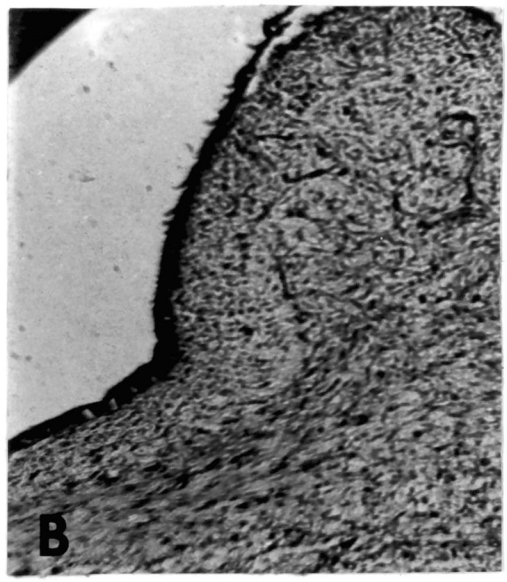
Fig. C. Control comb of a 13-day (stage 39) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 200.

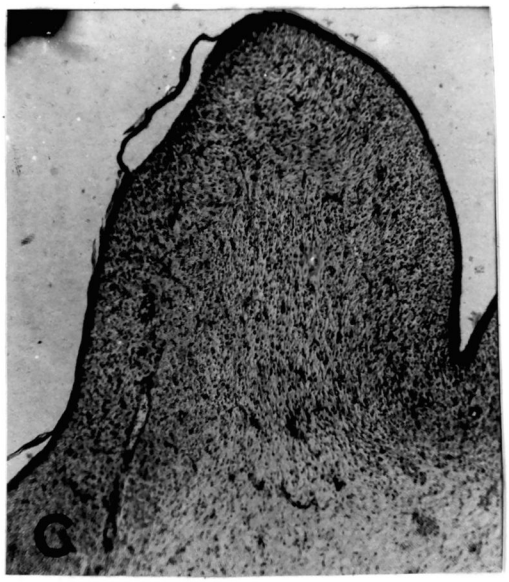
Fig. D. Experimental comb of a 13-day (stage 39) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 200.

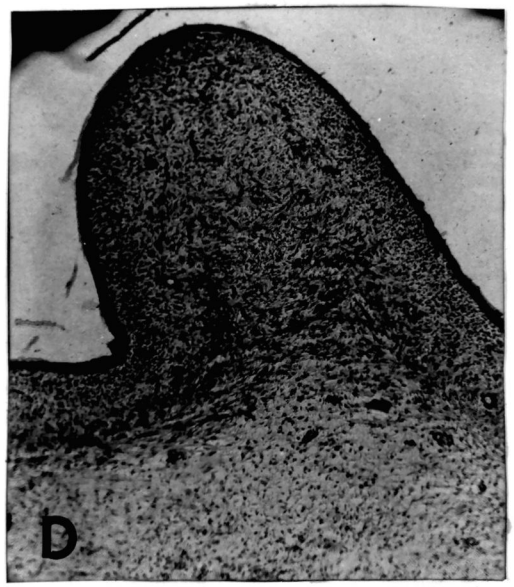
Fig. E. Control comb of an 18-day (stage 44) chick embryo. The cross section was taken in the anterior region of the comb ridge. Magnification: X 100.

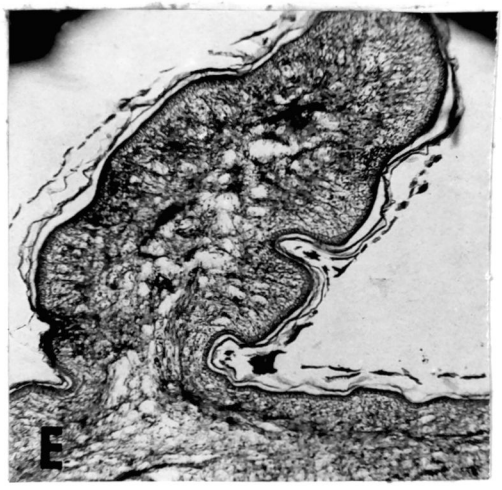
Fig. F. Experimental comb of an 18-day (stage 44) chick embryo. The cross section was taken in the anterior region of the comb ridge. Magnification: X 100.


A


B


C


D


E

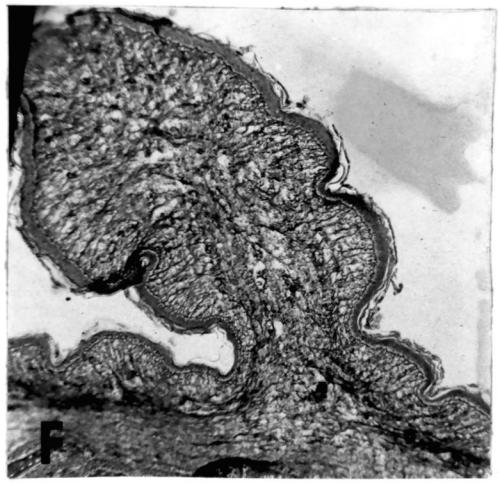

F

PLATE IV

All figures in Plate IV are of comb tissue sectioned at 10 microns and stained with the Alcian Blue 8GX schedule. The regions represented lie medial and basal to the junction between the comb elevation and the lateral comb areas.

Fig. A. Control comb of a 12-day (stage 38) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 440.

Fig. B. Experimental comb of a 12-day (stage 38) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 440.

Fig. C. Control comb of a 14-day (stage 40) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 440.

Fig. D. Experimental comb of a 14-day (stage 40) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 440.

Fig. E. Control comb of a 16-day (stage 42) chick embryo. The cross section was taken in the posterior region of the comb ridge. Magnification: X 440.

Fig. F. Experimental comb of a 16-day (stage 42) chick embryo. The cross section was taken in the posterior region of the comb ridge. Magnification: X 440.

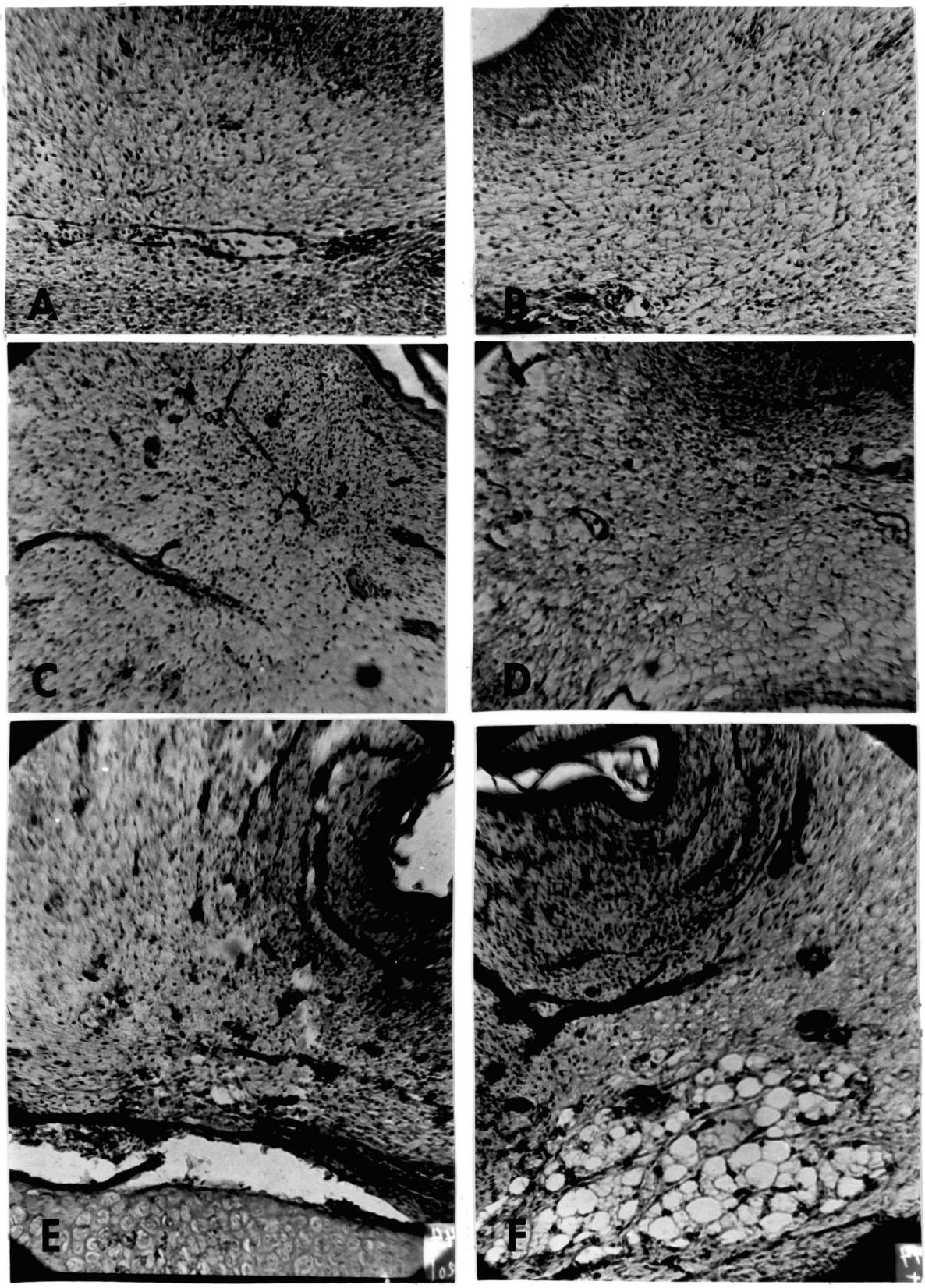

A
B
C
D
E
F

PLATE V

All figures in Plate V are of comb tissue sectioned at 10 microns and stained with the Hale Colloidal Iron-PAS technique.

Fig. A. Control comb of an 8½-9-day (stage 35) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 100.

Fig. B. Experimental comb of an 8½-9-day (stage 35) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 100.

Fig. C. Control comb of a 10-day (stage 36) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 100.

Fig. D. Experimental comb of a 10-day (stage 36) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 100.

Fig. E. Control comb of a 13-day (stage 39) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 100.

Fig. F. Experimental comb of a 13-day (stage 39) chick embryo. The cross section was taken near the middle of the comb ridge. Magnification: X 100.

Legend: E epithelium
B membranous bone
C ethmoid cartilage

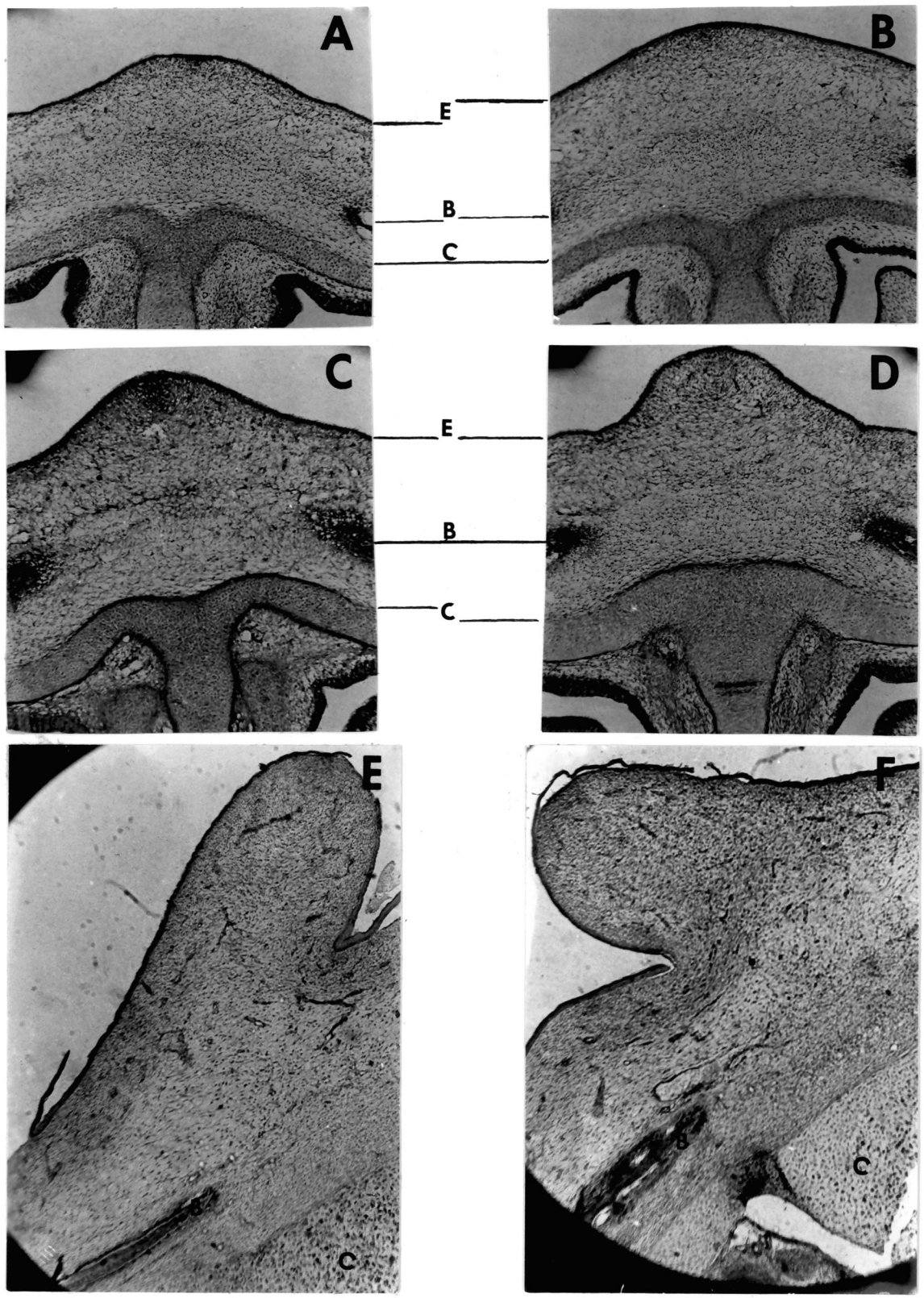

A
E
B
C
B
C
D
E
B
C
E
C
F
B
C

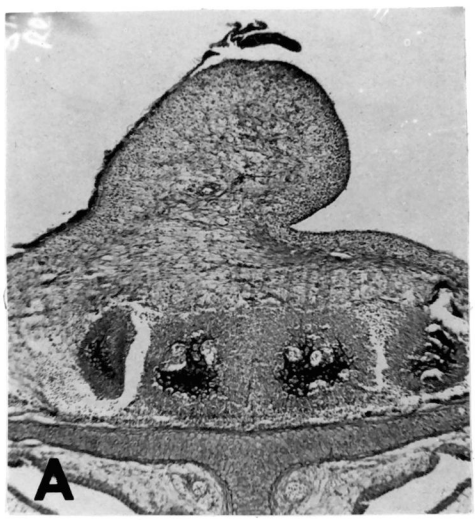
PLATE VI

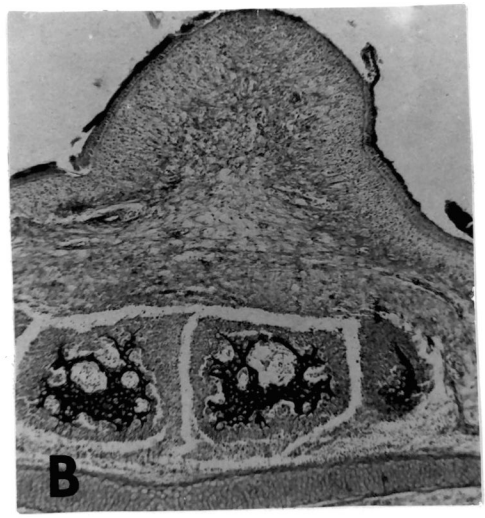
All figures in Plate VI are of comb tissues sectioned at 10 microns and stained with the Hale Colloidal Iron-PAS technique.

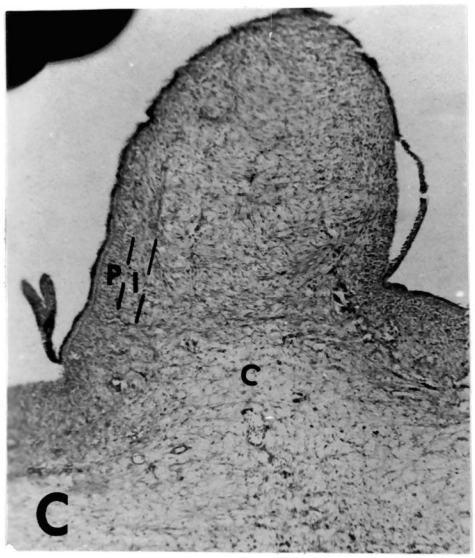
Figures A, C, and E represent anterior, medial, and posterior comb regions of a control 14-day (stage 40) chick embryo.
Magnification: X 100.

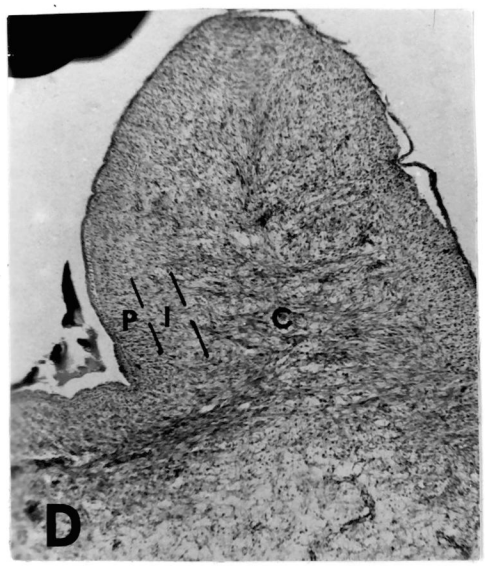
Figures B, D, and F represent anterior, medial, and posterior comb regions of an experimental 14-day (stage 40) chick embryo.
Magnification: X 100.

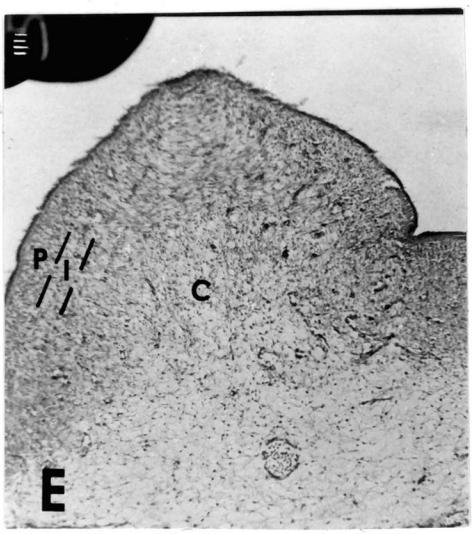
Legend: P peripheral dermal layer
I intermediate dermal layer
C central dermal layer


A


B


P
I
C
C


P
I
C
D


P
I
C
E

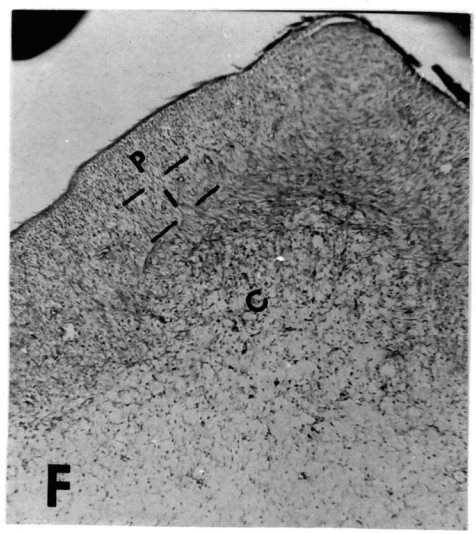

P
I
C
F

PLATE VII

Graphic Reconstructions

Fig. A. Longitudinal view of 12-day (stage 38) experimental and control combs. The widths measured at the base of the comb elevation are superimposed.

Fig. B. Lateral view of 12-day (stage 38) experimental and control combs. Heights of comb elevation are superimposed.

Fig. C. Longitudinal view of 14-day (stage 40) experimental and control combs. The widths measured at the base of the comb elevation are superimposed.

Fig. D. Lateral view of 14-day (stage 40) experimental and control combs. Heights of comb elevation are superimposed.

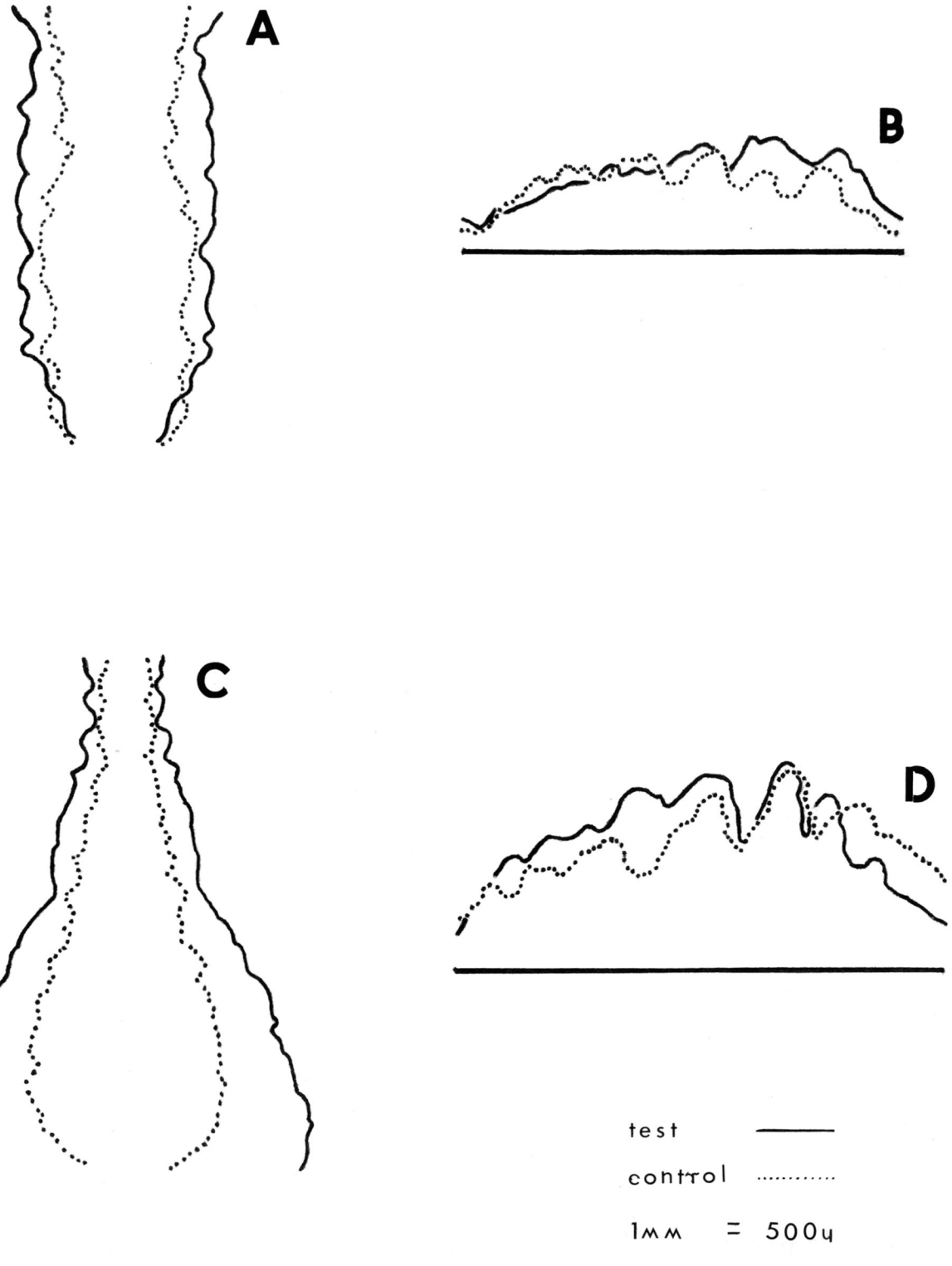

A
B
C
D
test
control
1mm = 500μ

PLATE VIII

Graphic Reconstructions

Fig. A. Longitudinal view of 16-day (stage 42) experimental and control combs. The widths measured at the base of the comb elevation are superimposed.

Fig. B. Lateral view of 16-day (stage 42) experimental and control combs. Heights of comb elevation are superimposed.

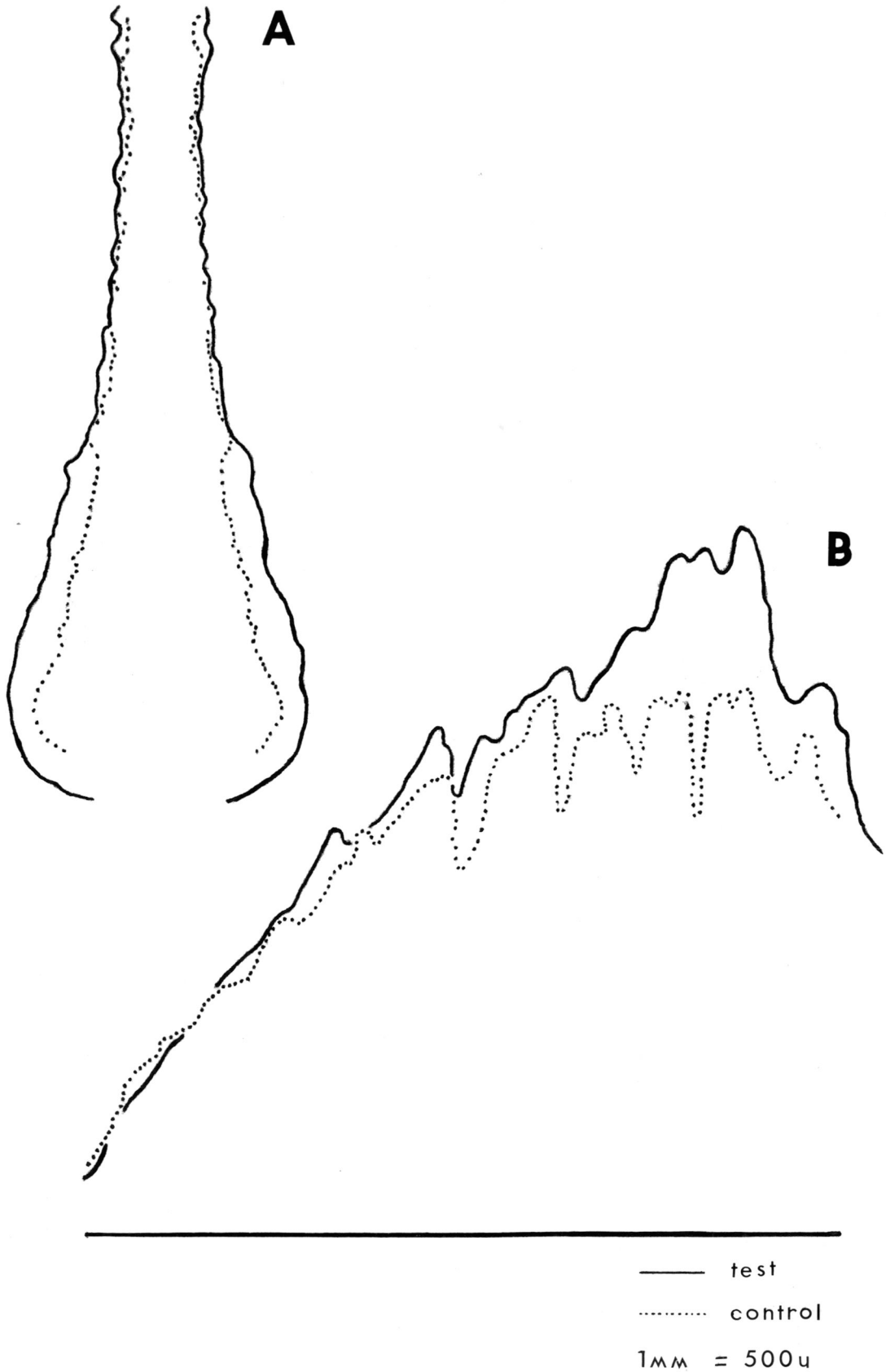

A
B
test
control
1mm = 500u

PLATE IX

Each figure indicates the concentration of an intracellular (figure A) or intercellular (figures B, C, and D) substance seen in developing testosterone treated and control combs. The ordinates indicate the observed concentrations of these substances at the developmental stages represented by the abscissas.

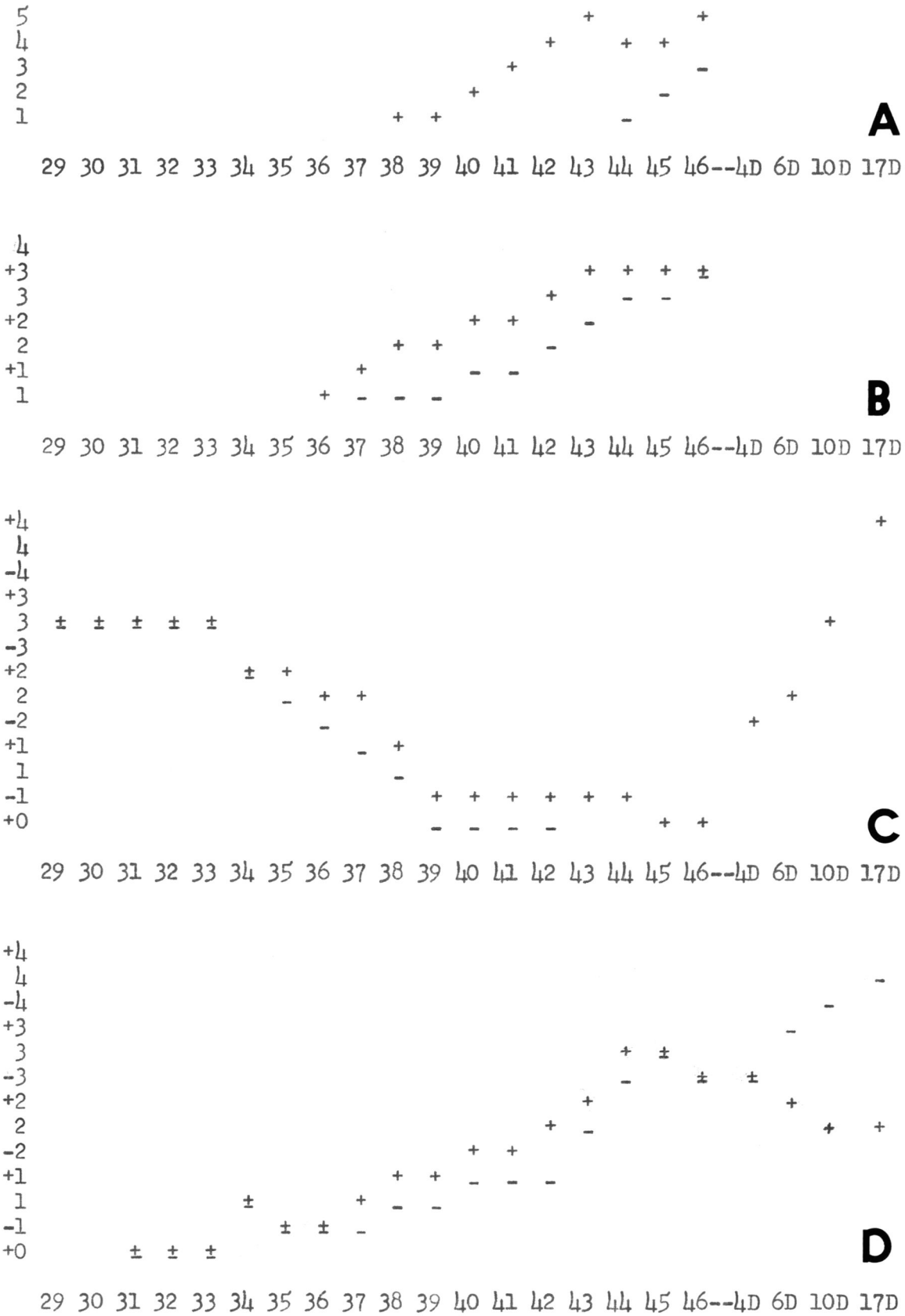
Fig. A. Lipoidal accumulation in adipose tissue cells. Comb tissue analysed from stage 29 to stage 46 (hatching) using the Alcian Blue 8GX Schedule.

Fig. B. Collagen assimilation by fibroblasts of comb dermal layers. Comb tissue analysed from stage 29 to stage 46 (hatching) using the Gomori Trichrome Technique.

Fig. C. Patterns of Hale positive mucopolysaccharide assimilation by fibroblasts of comb dermal layers. Comb tissue analysed from stage 29 to stage 46, and during the 4th, 6th, 10th, and 17th days of post embryonic development with the Hale Colloidal Iron-PAS technique.

Fig. D. Patterns of Hale negative mucopolysaccharide assimilation by fibroblasts of comb dermal layers. Comb tissue analysed from stage 29 to stage 46, and during the 4th, 6th, 10th, and 17th days of post embryonic development with the Hale Colloidal Iron-PAS technique.

Legend: + concentration of specifically stained cellular product in testosterone treated comb
- concentration of specifically stained cellular product in control comb


A
B
C
D
29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46--4D 6D 10D 17D