1955

Histochemical localization of certain constituents of the developing juvenile wing feather

Alice Louise Koning

Iowa State College

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HISTOCHEMICAL LOCALIZATION OF CERTAIN
CONSTITUENTS OF THE DEVELOPING
JUVENILE WING FEATHER

by

Alice Louise Koning

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Embryology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

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Dean of Graduate College

Iowa State College

1955
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INTRODUCTION

During the course of a previous study on the histochemistry of the down feather (Koning and Hamilton, 1954), it was found that the early appearance of alkaline phosphatase in the dermal core of the feather, and its high concentration in the formative zone at the base of the feather during its period of growth and differentiation suggested that this enzyme might function as a mediator of tissue differentiation. A concentration of ribonucleic acid in the basal layers of the epidermis, in juxtaposition to the dermal phosphatase, implied a physiological connection between the two substances. The significance of the polysaccharides in the pulp and glycogen in the barb ridges was also considered. Since the down feather is the first and simplest of several generations of feathers to arise from the same follicle in the skin, it seemed that perhaps a greater understanding of these findings could be gained by extending the study to include the second or juvenile feather generation.

Basically, the down and juvenile feathers are composed of similar morphological units, viz., barbs, barbules, and the calamus or quill; but the juvenile feather is much more complex both in the refinements of these units and in the embryonic organization of the papilla from which they are formed. First, the juvenile feather, unlike the down feather, which arises from previously undifferentiated tissues, originates in a well-defined follicle and from tissues which have already given rise to one feather. Second, the down feather is radially symmetrical, consisting of a conical
epidermal cylinder from which a series of longitudinal barb ridges arises practically simultaneously. The juvenile feather, by contrast, is distinctly bilateral, although it retains a circular base. Barbs form continually over an extended period, arising on the ventral side of the circular base and lengthening as they pass dorsally around the cylinder to unite with the dorsal shaft or rhachis. The rhachis itself extends distally, carrying the barbs with it. There is, then, a bilaterally symmetrical gradient imposed upon the original radial symmetry of the feather. The form of the symmetry and the initiation of the epidermal pattern are known to arise by the inductive activity of the dermal papilla (see literature review below). There is also the probability that structural details of the epidermal feather such as barbs, barbules, and hooklets are dependent upon the mesodermal substratum (Hamilton and Koning, 1954).

From the foregoing, it appears clear that the juvenile feather possesses additional refinements of structure and physiological and developmental gradients which make it particularly attractive for histochecmical and embryological studies. By a comparison of the histochecmical changes in the juvenile feather with those found in the down feather, it is hoped that the present study may reveal some of the morphogenetic processes common to both.
Although earlier workers had attempted to describe the development of the feathers, studies have shown that the feathers are derivatives of the dermal component of the follicle, from which the epidermal cells of the feather are derived. The dermal component of the follicle forms the cortex, from which the outer part of the keratinous shaft of the feather is derived. The outer part of the keratinous shaft is composed of the outer layer of the epidermis, which is the outermost cell layer. The inner part of the keratinous shaft is composed of the intermediate cell layer, which is connected between the outermost and inner layers, and appears to originate from the junction of the epidermis with the dermis.

Upon reaching the epidermis, the inner layer consists of the stratum corneum layer of the skin. The inner cell layer consists of the stratum corneum layer of the skin and within the keratinization layer of epidermal cells to form the complete outermost layer of cells. The outermost is a dome-shaped component consisting of those layers of cells that are in contact with the epidermis. The outermost is a similar component to the inner surface of the epidermis, where it is in contact with the dermis of the feather base. The outermost is a similar component to the inner surface of the epidermis, where it is in contact with the dermis of the feather base.
downward. In the base of the follicle is the dermal papilla, from which
the mesodermal core of the feather arises.

The formation of barb ridges has nearly or completely ceased with the
formation of the quill of the down feather; however, it begins anew as the
elements of the definitive feather are formed. The walls of the juvenile
feather cylinder are thick in contrast to the relatively thin walls of the
down feather. Instead of barb ridges forming as thickenings in the wall of
the feather, they are cut out from the cylinder by longitudinal infolding
of the stratum cylindricum, which thus separates the cylinder into a series
of parallel ridges. These ridges form almost simultaneously around the
feather cylinder, except for a small area on its dorsal surface. There,
the rhachis arises and projects distally by the progressive union of ridges
on either side of this point. Additional barb ridges continue to arise
ventrally on either side of a small triangular area (the ventral locus),
and pass dorsally with oblique orientation around the collar to the rhachis.

Further differentiation of the barb ridges occurs chiefly in the
stratum intermedium. On either side of the ridge, the cells line up to
form a row of cylindrical cells, which make up the barbule cells of the
barb plate. At the inner edge of the ridge is a central mass of undiffer-
entiated cells making up the barb stem. These cells enlarge and hollow
out; later cornification leaves only the rigid cell walls apposed to each
other. These cells become the medullary substance of the barb stem upon
which other cells condense and cornify to form the cortex of the barb.

The cells making up the barbule plate elongate peripherally and
distally at their inner edge, with the cells nearest the barb stem under-
going the greatest displacement. These latter cells form the attachment
of the barbule to the barb; all of the cells of the barbule plate then
cornify to complete the formation of the barbule.

As the feather is completed, the pulp begins to shrink back towards
the base of the feather, forming a series of pulp caps as it retreats.
At the completion of development the pulp is no longer present in the
feather except for the dermal papilla at its base. When the feather is
lost, the papilla remains behind, eventually regenerating another feather.

Davies did not clearly state what might be the most active cell layer
involved in the formation of barb ridges. Strong (1902), however, in his
work on the origin of color in feathers, inclined to the opinion that
neither the stratum cylindricum nor the dermis forces the alignment of the
stratum intermedium into ridges. Instead, he thought that the cell move-
ments involved in the formation of ridges are intrinsic in the stratum
intermedium, the cylindricum and pulp merely following the course set for
them after the ridges have formed.

Davies described the juvenile feather as a structure separate from the
down feather. Riddle (1908), however, postulated that the down and juvenile
feathers are one and the same structure. He believed that the quill of the
down feather is formed as a result of the poor nutritional status of the
bird after hatching, as well as the effect of chilling on the blood supply.
This theory has proven untenable on the basis of actual calculated growth
rates as well as the obvious fact that quill formation in down feathers
occurs at different times, both before and after hatching, depending on the
location of the feathers.

In 1932 Lillie and Juhn reported on some of the mechanics which are
involved in forming the structure of the definitive feather. They observed the strict seriation of barbs, i.e., that each barb is formed sequentially from apex to base, the distal barbs first, followed in order by the next proximal barbs. The barbs arise at the ventral side of the feather and pass around the collar toward the dorsal side, converging to form the rhachis. They found a distinct gradient of growth in barbs, with the apex of the barb being formed more rapidly, and proximal portions relatively slowly.

In 1936 Lillie and Juhr presented further results which confirmed most of the conclusions of the 1932 paper, at the same time making some revisions. They found that only the lateral parts of the rhachis are formed by the series of barbs as they pass around the collar from their point of origin. The central part of the shaft is composed of cells originating presumably from the intermediate cells of the ramogenous (barb-forming) zone lying just above the collar. There are three vectors of growth occurring simultaneously in the feather. One is radial growth, resulting in an increase in the diameter of the feather. Another is axial elongation, which occurs in the sheath cells and in the intermediate layer of the epidermis with its developing barbs and barbules as both layers originate from the collar. Thirdly, a tangential growth combined with the axial growth causes the base of each slanted barb, as its apex passes distally from its ventral point of origin, to move dorsally around the rim of the collar until it reaches the rhachis, at which time the barb has attained its full length. Lillie and Juhr also found that the epithelial surface next to the pulp in the area where the barbs are just forming appears to be devoid of a basement membrane.
Most of the studies mentioned above were concerned almost exclusively with the specializations of the epidermis and formation of the barbs. Davies (1889) referred to the dermal papilla and pulp briefly, and mentioned the formation of pulp caps at the end of the period of growth of the feather. Lillie (1940) in a more complete study, found no evidence of cell division in the pulp above the dermal papilla. From the relations of the pulp to the epidermis, he determined that the pulp is supplied by the papilla at its base and grows at the same rate as the epidermis. However, after a certain "functional" length is attained, the pulp appears to be absorbed apically, its tip remaining at a constant distance above the base of the feather. A series of pulp caps is formed apically from the inner layer of the stratum cylindricum, the so-called pulp membrane, as a result of absorption and shrinkage of the pulp below it. Lillie emphasized the importance of the pulp from a nutritive standpoint; the rapidly growing epidermis needs a good blood supply. The thinning and seeming disappearance of the basement membrane in the region of the ramogenous zone may possibly facilitate the absorption of nutrients. The pulp is also important in maintaining the rigidity of the feather and preventing distortion of the epidermal elements until they are sufficiently keratinized to support themselves. In this connection, the pulp maintains its turgor by means of a gelatinous, homogeneous ground substance. The pulp cap is important in protecting the pulp from desiccation and maintaining the water gradient of the feather.

Goff (1949) also studied the mesodermal constituents of the feather in chick embryos, with particular emphasis on the blood supply to the pulp. By injecting cutaneous blood vessels in the wings of chick embryos,
he observed that at about 11 to 15 days of incubation the longitudinal system of blood vessels that has been developed in the down feather changes to a dispersed network of vessels which indicates the first organization of the juvenile feather.

Experimental studies

The conspicuous sexual dimorphism both in color and structure of the plumage in many species of birds led to experimental studies of the effects of hormones and their correlations with growth rates within the feather. In general it was found that the threshold of response to a hormone is directly proportional to the rate of growth of the feather (Lillie and Juhn, 1932). The most clearly defined growth gradient appears in the barbs, with growth decreasing from the apex to the base. In addition there is also a disto-proximal gradient in the entire feather, with the low point in the proximal part of the feather.

By means of operative techniques involving defects, reorientation, and transplantation, Lillie and Wang (1940, 1941) found that (1) the orientation and tract-specific morphology of the feather (i.e., saddle, breast, etc.) are a function of the entire papilla; (2) the dorsal half of the papilla is needed for the formation of a rhachis, but barbs form whether or not the rhachis is present; (3) papillae placed in the featherless areas of the skin tend to organize a feather at the new site; (4) half-papillae of two feather types can produce either twin feathers or unite to form a chimaera. These various anomalies persist through succeeding generations of feathers from altered follicles.

Additional information on the morphogenetic activity of the components
of the papilla was reported by Wang (1942, 1943). He destroyed the epi-
dermal covering of the papilla by the use of salicylic acid in a collodion
solution. These demuded papillae were then placed in empty follicles of
various feather tracts. Epidermis from the host follicle covered the
demuded papilla and differentiated into a feather. Wang found that the
morphology of the feather is a function of the epidermis from which it
is derived, but that its orientation and symmetry are controlled by the
dermal component. Furthermore, the ability to form feathers is not limited
to the epidermis at the base of the follicle, but extends for a short
distance along the follicular walls. Epidermis from the upper parts of
the follicle and from the surface skin is unable to form feathers in the
presence of a demuded papilla. Wang emphasized the importance of the
dermal papilla as a typical embryonic field. Although it is the activating
factor, it needs competent epidermal tissue on which to act, and the
response of the epidermis is in accordance with the nature of the tract in
which it is located.

By combining dorsal and ventral halves of feather papillae from
different tracts, Lillie and Wang (1942, 1943) were able to localize the
areas in the papilla which govern the characteristic pattern of the
feather. In these experiments they used combinations of breast and saddle
papillae. They found that while growth rate is determined by the dorsal
component alone, the total length of the feather and the extent of the
fluff at its base are influenced by both components.

In further elaborations of the above experiment, Lillie and Wang (1944)
implanted dorsal and ventral sectors of either breast or saddle papillae
into saddle or breast papillae dorsally or ventrally, to give a variety of
combinations. They concluded that the capacity for inducing the rhachis lies exclusively in the dorsal half of the papilla, being strongest at the dorsal center of the collar and grading off bilaterally. Splitting of the dorsal field causes a reorganization of each segment and the formation of twin feathers. Apparently the induction is carried out by means of a contact effect of the papilla on the substratum, i.e., the epidermis of the collar region. If the dorsal field is weakened, as occurs late in development or else by an increase in substratum, the ventral barb-forming field escapes from its influence and forms an after-feather by a process of twinning. The activity of the papilla, therefore, appears to be two-fold (Lillie, 1942). It acts in the induction of the rhachis, after which its morphogenetic function seems to be accomplished. In addition, the papilla continually forms pulp to support the feather throughout its regeneration.

The evidence for tract-specificity of the ectodermal response to the general inductive action of the dermal papilla does not apply to the juvenile feather. In 1947 Saunders first reported that in chick embryos the transplantation of leg tissue to wings showed thigh feathers forming in some cases even after the prior removal of the leg epidermis. This was later confirmed by Saunders (1951) and also independently by Cairns (1951) (also Cairns and Saunders, 1954). In their experiments they transplanted thigh mesoderm that had been freed completely of its ectoderm into the wing of a host embryo. In a majority of cases they found supernumerary feathers on the wing, either of wing or leg feather morphology. There were no feathers of an intermediate type. In another series of experiments the transplantation of mesodermal leg tips beneath the apical ectoderm of wings
produced abnormal wings terminated with structures bearing characteristic
leg armament, e.g., claws and scales. They concluded that at this early
stage the mesoderm does appear to act in a specific manner and can establish
specificity in the ectoderm which is retained throughout the life of the
bird. However, they found some modification of this effect by the wing
environment.

Histochemistry

An extensive review of the literature in connection with the histo-
chemistry of the epidermis and epidermal derivatives has already been made
(Koning, 1953), and only a few of the more pertinent studies need be men-
tioned here.

Bourne (1943) was the first to find a phosphatase reaction in the
follicle of the hair, particularly in the central part of the sheath; he
observed little reaction at the bottom of the follicle. Johnson, Butcher,
and Bevelander (1945) reported that phosphatase is present in the connective
tissue sheath of rat hair and also in the papilla, beginning with very early
stages of development and nearly disappearing during the resting stage.
They suggested that phosphatase might be correlated with cellular prolifera-
tion and histo-differentiation.

Montagna, Chase, and Lobitz (1952) examined the skin and hair for
cellular polysaccharides. They found that glycogen is present in the external
sheath of hair follicles in the form of intercellular bridges, as well as
in the internal sheath and the medulla of slowly growing hairs. Glycogen
appears to be abundant in sites where a damming of keratinized material
has retarded the normal rate of keratinization. The glycogen disappears
as keratinization progresses. In another study, Montagna, Chase, Malone, and Melaragno (1952) found that the dermal papilla shows the presence of polysaccharides on the fourth day after the initiation of growth in the follicle, at a stage in development immediately preceding proliferation of the hair shaft. These polysaccharides react metachromatically to toluidine blue, and are also reactive to the periodic acid-Schiff test for diglycols; for this reason these workers postulated the presence of at least two different polysaccharides in the papilla. These materials remain in the papilla until just before the onset of the resting stage, when both reactions cease abruptly.

Hardy (1952) studied the composition of the hair follicles of the mouse, utilizing most of the major histochemical techniques. She found that ribonucleic acid is abundant in the hair matrix, central cortical zones, and some regions of the inner and outer root sheath. Glycogen is present in the outer root sheath and medulla, and other polysaccharides are found in the dermis. Alkaline phosphatase is present in the papilla, especially in the early stages of rapid growth, in most of the hair matrix cells, and in the outer root sheath. Sulphydryl is present in the upper levels of the cortex and cuticle; disulfide occurs in the most distal cortical zones.

It should be emphasized here that the hair and feather are not homologous structures. These results have been presented since hair is an epidermal specialization, which, although not of the same derivation as the feather, is a keratinized structure with a papilla at its base. For this reason it might be instructive to determine its histochemical nature
and compare it with that of the feather, since a duplication of substances existing in the two might implicate some of them in the process of keratinization.

The histochemistry of feathers has not been studied by many workers. Giroud and Bailliard (1930), using a nitroprusside technique for sulfhydryl, observed the feather sheath to be keratinized at its base, with the barbs keratinizing later at about the level of the surface skin. Johnson and Bevelander (1947a) studied the localization of alkaline phosphatase and glycogen in the down feather of the chick. They found phosphatase present from the ninth day of incubation onward, first in the dermal papilla, and later not only in the papilla but to some extent in the basal layers of the epidermis and in the membrane separating the barb ridges from the pulp. Glycogen appears at 13 days of incubation in the cells of the barb ridges, disappearing four days later. They connected the phosphatase with sites of rapid growth and specialization of tissues.

Moog and Wengé (1952) studied the comparative localizations of alkaline phosphatase and a neutral mucopolysaccharide in a wide variety of organs. They observed that the dermal elements of the down feathers are weakly positive in both cases.

Koning and Hamilton (1954) studied the down feather of the chick histochemically. They found that alkaline phosphatase appears in the early stages of the condensation of the mesenchyme to form the dermal papilla. The reaction is weak at first, increasing rapidly just above the base of the papilla, both in the mesenchymal cells and intercellular fibers. It becomes especially active along the basement membrane, and is
differentially localized on the dorsal and ventral sides of the papilla. Little epidermal phosphatase is found, with the exception of the basal layers near the proximal end of the feather. Ribonucleic acid is present in the cytoplasm of the epidermal cells which lie adjacent to the most highly phosphatase-positive dermis. Glycogen appears in the more fully formed barb ridges, being located in the central cells of these ridges. A metachromatic polysaccharide is present in the fibrillar matrix of the pulp. A small amount of periodic acid–Schiff reactive polysaccharide is also found in the pulp. Protein is present especially in the epidermis and barb ridges, and also appears in fibers in the basal pulp.

No studies on the definitive feather have appeared, except for one preliminary abstract of the present work (Koning, 1954).
MICRONS, FREEZE SECTION AT 10 TO 15 MICROMETERS

AIDEN) WITH SECTIONED, PARAFFIN-EMBEDDED TISSUE WAS SECTIONED AT 5
CUTS. THE BLOCKS OF GELATIN WERE HARDENED IN BAKER'S FORMAMIDHE-JODACETATE-CATIONS. FLUID
FOR 18 TO 20 HOURS AND EMBEDDED IN 25 PERCENT GELATIN. THE BLOCKS

FEETHERS WERE FIXED IN BAKER'S FORMAMIDHE-JODACETATE-GERMREXITE (1:17:1)

FOR FREEZE SECTION.

AIDEN) CONTAINING 10 PERCENT BEESWAX. FOR FREEZE SECTION

THE GELATIN-FIXED SECTION WAS EMBEDDED IN 25 PERCENT G/P (P. 52
AND EXTRAVASCULAR TISSUE SURROUNDING THEM.

THE SECTIONING, AND THE JUVENILE FEETHERS WERE DIVIDED OUT OF THE BONE

LATER THE DOWN FEETHERS WERE REMOVED AS COMPLETELY AS POSSIBLE TO AID IN

FOR THE REST OF THE FEETHERS, THE WHOLE WING WAS FIXED AND SECTIONED.

FEETHERS ON THE POSTERIOR EDGE OF THE WING COVERS WERE NOT YET FORMING.

AFTER HARVESTING (T. 90 TO THE, HAMMURABII AND HAMMERSON, 1957), AND FOR TWO DAYS

EMBRYO OF DAY 7, FROM THE MID-REGION OF THE THORACIC TEGMENTUM WITH

FEETHERS FOR THE STUDY WERE OBTAINED FROM THE MOUTH OF WHITE LEGHORN

GENERAL PROCEDURES

MATERIALS AND METHODS
Histochemical Procedures

**Alkaline phosphatase**

A modification of Gomori's (1939) original technique was used. The substrate contained 2 percent solutions of the following: 20 ml. of sodium barbital, 20 ml. sodium glycerophosphate, 10 ml. calcium chloride, and 2 ml. magnesium sulfate, to which was added 50 ml. of water. In the control substrate, water replaced the glycerophosphate. The feathers were incubated for 1 hour in either the substrate or the control, washed, treated with 2 percent cobalt nitrate, washed again, and the color developed by means of a dilute solution of ammonium polysulfide.

**Ribonucleic acid**

Sections were stained in a solution of 0.05 percent toluidine blue in 5 percent alcohol for 15 minutes. Due to the basophilia of the nucleic acids, the tissue stains heavily. Previous to staining, some sections were incubated in a 0.1 percent solution of ribonuclease in distilled water for 2 hours at 50°C, while others were incubated for the same length of time in distilled water alone. Structures which lost their basophilia after this treatment were presumed to have contained ribonucleic acid. In all cases differentiation and dehydration were carried out overnight in tertiary butyl alcohol (Montagna, Chase, Malone, and Melaragno, 1952). From the alcohol the slides were cleared in xylol and mounted in H.S.R. mounting medium.

**Polysaccharides**

For a wide variety of polysaccharides, the periodic acid-Schiff (PAS) reaction (McManus, 1946; Hotchkiss, 1948) was used, following the
procedure given by Lillie (1954). The sections were counterstained with Mayer's acid hemalum for three minutes and its blue color developed in sodium carbonate. The polysaccharides stained varying shades of red or pink, the nuclei blue.

Acid mucopolysaccharides were further identified by their ability to stain metachromatically with basic dyes. In the sections treated with toluidine blue, these substances stained various shades of pink.

Glycogen was stained by the PAS reaction. Its identification was confirmed by treating control sections with saliva for 30 minutes at 37° C, immediately preceding the PAS reaction. Loss of staining indicated which areas had previously contained glycogen.

Proteins

For a study of the overall distribution of proteins, the mercuric chloride-bromphenol blue (Hg-BPB) technique of Mazia, Brewer, and Alfert (1952) was used. Sections were stained for 15 minutes in an alcoholic solution of bromphenol blue and mercuric chloride, then washed for 20 minutes in 0.5 percent acetic acid, followed by (1) development of color in water for 2 minutes, dehydration and mounting, or (2) several changes of tertiary butyl alcohol until the excess dye was removed and the tissue dehydrated, followed by mounting. Although the first procedure gave the best color, the loss of dye was often considerable, and the two methods were utilized to check on the amount of color loss.

Various methods were used in an attempt to demonstrate the presence of keratin and/or disulfide bonds. One method consisted of alkaline hydrolysis and staining with blue tetrazolium (Pearse, 1953). Sections
were incubated at 60° C in a solution of blue tetrazolium in an alkaline buffer, pH 12.8, for 2 to 4 hours. They were then washed and mounted in glycerine jelly. Sites of disulfide bonds were colored light blue due to the reduction of the tetrazolium by hydrolysis products of the disulfide linkages.

Another method consisted of oxidation by means of peracetic acid and subsequent staining with azure and eosin (Illie, 1954). The oxidation of the disulfide linkage causes the production of cysteic acid and thus increases the capacity to take up basic dyes. The oxidation by peracetic acid gave poor results in our hands. Potassium permanganate (0.5 percent) was tried as an oxidizing agent (Ilie, Banglie, and Fisher, 1954) and proved more satisfactory. The sections were first collodionized (1 percent collodion) and then oxidized in the permanganate for 20 to 25 minutes. They were rinsed in oxalic acid (1 percent) just long enough to remove most of the brown crystals and then washed for 10 minutes in running tap water. They were stained for 1 hour in 0.05 percent methylene blue in 0.1 N HCl. The sections were dehydrated in acetone directly from the stain, cleared and mounted.

**Lipids**

Sudan black B was used as a stain for lipids. A saturated solution (about 0.1 percent) in 70 percent alcohol was made up, and frozen sections were stained in it for 7 minutes. After rinsing in 50 percent alcohol, the sections were washed in water and mounted in glycerine jelly. Some sections were also counterstained in Mayer's carmalum before mounting, to reveal the nuclei.
OBSERVATIONS

Papilla and Pulp

At 13 days of incubation, the juvenile feather has just begun to form, judging from the increased diameter of the feather at its base, but it is not until 24 to 48 hours later that the first barb ridges appear. At this stage the dermal papilla of the most advanced wing feather is not clearly defined, consisting only of a condensation of mesenchymal cells at the base of the follicle.

During these early stages, alkaline phosphatase is reactive in the cells and fibers of the pulp throughout its length. The enzyme is present adjacent to the epidermal tissue down to the base of the feather, lying below the level of the base only at the sides, beneath the epidermis. In the center of the feather cylinder, the enzyme does not extend beneath the level of the base. The entire lower limit of its activity, therefore, takes on the appearance of an inverted cone (Fig. 2). This is in sharp contrast with the subgerminal gradient of phosphatase in the down feather (Fig. 1).

Ribonucleic acid (RNA) is already present in the cells of the dermis at 14 to 15 days. The cells nearest the epidermis show the strongest reaction, although some of the increased staining is due to a concentration of dark-staining nuclei at this point. The mercuric chloride-bromphenol blue (Hg-BPB) stain for proteins reveals a condensation of heavy fibers and cells at the proximal end of the condensed dermal tissue which marks
definitely, but the differentiation to much less marked in younger stages.

Furthermore, in the most fully differentiated portions these areas are characterized by solid columns of protophase-reactive nuclei that extend up the leathery surface of the epidermis. These two areas unite the protophase-A of the epidermis to form a peripheral layer of the pupa. Some protophase areas appear in the periphery and proceed towards the periphery of the region shown in the last two figures (5 and 6). Here the leathery area, nearly to the level of the collar (figs. 2 and 3). This is first there in considerable areas around the axilla and proceeds to the outer part of the collar.

Closely the protophase activity is limited to the pupa with the development (fig. 6).

This "the" areas of the early showing protophase at different stages can be followed in the series of figures showing protophase at different stages. These changes can be traced to the differentiation of the epidermis and protophase differentiation from the cuticle. Taking protophase differentiation from the epidermis, especially at the base of the collar, and proceed towards the outer part of the collar, the cuticle of the region of the collar of the pupa.

On the other side, the region of the collar and the base of the collar, the pupa and both extracellular, because present in the wall and in the ground substance, both extracellular, because present on the pupa, and to the papilla. Most of the material in the region of the base of the collar, reacting as electron-dense.
In sections stained for phosphatase, one of the noticeable features of the pulp at nearly all levels of the feather is its close adherence to the epidermis. This becomes particularly evident in the ramogenous zone (Fig. 7). There is a narrow band of one or two layers of cells and fibers lying against the epidermis, which assumes the appearance of a basement membrane. This layer is often separated partially from the remaining pulp by a series of cavities lying in the periphery of the pulp. These may be blood sinuses, although no blood cells are found in them. More likely, they are artifacts produced during fixation by the shrinkage of the pulp. The connection between the epidermis and its adjacent pulp must be very firm in this case, since this portion of the pulp adheres to the epidermis in preference to the remainder of the pulp.

At the level at which the barb ridges begin to form, the greatest accumulation of phosphatase is in the center of the pulp. Although the peripheral pulp adheres closely to the epidermis, it is not ordinarily highly reactive, and the formation of the ridges seems to take place without the intervention of phosphatase. Above the level at which the barb ridges originate, phosphatase becomes very active in the adjacent pulp and pulp extensions which lie between each ridge (Figs. 8 and 9). This high level of phosphatase activity continues in the pulp next to the epidermis until the barbule cells have completed their differentiation, at which time the extensions of the pulp begin to pull away from the epidermis and shrink back to the central pulp cavity.

Ribonucleic acid is present in all of the cells of the pulp, especially near the collar (Fig. 14). As the papilla develops, the nuclei of its cells become less basophilic, but the cytoplasm retains its basophilic
and even increases it somewhat. The cells are closely packed and the cell boundaries are difficult or impossible to identify. In the rest of the pulp, the nuclei are highly basophilic and smaller than those in the cells of the papilla.

Metachromatic mucopolysaccharides, which in early stages are present throughout the pulp, are lost from the papilla as it differentiates. They are present in the dermis of the follicular wall and pass around the collar in a narrow band of the dermis and up into the pulp of the feather, where they spread quickly throughout it. They are most highly concentrated just above the papilla, gradually fading out distally (Fig. 17). The metachromatic substance is intercellular, being found in the ground substance and on fibers.

Periodic acid-Schiff (PAS)-reactive polysaccharides of two types are found in the pulp. One type is present in the ground substance and intercellular fibers and possibly to a small extent in the cytoplasm of the cells. It is found in the papilla and pulp, lessening in amount in a proximo-distal direction. In addition, this same type of polysaccharide is present in great quantities in the dermis of the follicular wall throughout its length. The reaction is very striking because of its intensity and the fact that it is confined to a narrow band of the dermis lying adjacent to the follicular epidermis (Figs. 18 and 2h).

In addition to this unidentified polysaccharide, glycogen also appears in the pulp. At about 15 to 16 days, a few granules are present in the dermis of the feather just above the level of the collar. These granules accumulate slowly at first (Fig. 10), but after the papilla has become
well-defined at 19 days, they increase rapidly just above it until they can be found in large numbers (Fig. 11). Varying greatly in size, they are intracellular in some instances, but in other cases are found associated with the intercellular fibers at this level. After treatment with saliva or malt diastase they disappear completely.

The changes in the distribution of proteins as revealed by the Hg-BPB test are confined almost exclusively to the papilla. In the first four days of development of the feather, heavily-staining fibers are visible in the base of the papilla (Fig. 12). Gradually, at the end of this period, these fibers become numerous throughout the papilla, extending from the base distally (Fig. 13). In addition to these fibers, the cells of the papilla also stain deeply. The remainder of the pulp does not show any concentrated protein reaction. The disulfide tests fail to show any differential localizations of disulfide groups in the pulp.

The papilla remains consistently negative to the test for lipids during the course of its development. In the pulp itself, few cells stain in early stages, but beginning at about 17 days a number of cells begin to fill with large lipid-containing granules. These cells are distributed fairly evenly throughout the upper levels of the pulp, but decrease in number towards its base. The nucleus is completely negative, but the cytoplasm is filled with granules of varying sizes, most of them quite large. The cells do not resemble the ordinary dermal cells, which are elongate or stellate with numerous cytoplasmic processes. Instead, the lipid-containing cells are more or less round and have no processes. They have the general appearance of macrophages.
Epidermis

The development of the epidermis can best be described by considering its parts separately. First and perhaps most important are the collar region and ramogenous zone, from which the cells of the remaining parts of the feather, viz., the sheath, barbs, barbules, and rhachis, are derived. The sheath will be considered along with the collar and ramogenous zone; the other structures are dealt with in later sections.

**Collar and ramogenous zone**

Until about 17 days of incubation, the collar is a specific region of the feather in name only. It refers to the point at which the invaginated cuboidal layer of the epidermis making up the wall of the follicle turns back upon itself and becomes modified to form the wall of the feather. Between these two cell walls arise the sheath cells of both the follicle and the feather. During the first day or two of development, the layer of sheath cells has not yet split, but forms a solid sheet of cells separating the inner and outer layers of the epidermis (see Fig. 2). The collar region has begun to turn obliquely inward to form a constriction in the papilla at the base of the feather. This process continues during development and is accentuated by the great increase in width of the collar (compare Figs. 14 and 16).

At this early period in the development of the feather (14 to 16 days), histochemical differentiation of the epidermis has not proceeded far. Alkaline phosphatase is weakly reactive in the nuclei of the cells of the follicular wall and feather, but not the sheath cells (Fig. 3). Above the collar in the feather, the nuclei are slightly reactive in the cylindricum
and some of the cells of the intermedium. The cells of the wall of the
follicle and of the feather contain ribonucleic acid. In the cells of the
cylindrical layer, most of the RNA appears in their base, abutting the
dermis (compare Figs. 14 and 15). The sheath cells have considerable RNA,
but in contrast to the basophilic nuclei of the other two layers, their
nuclei are nearly colorless except for a prominent nucleolus. All of the
cells contain relatively high amounts of protein (Fig. 12). There is no
polysaccharide in the epidermis at this time, nor does any appear at any
later stage of development, except for glycogen in the medulla of the
rhachis and barbs (see below).

In the further development of the epidermis, phosphatase tends to
disappear from most of the cells. The nuclei of the follicular cells
show considerable activity, especially near the base of the feather (Fig.
3). Farther distally, these cells become very much flattened and lose
their activity. Passing around the collar and up to the ramogenous zone,
the nuclei of the basal layer contain some phosphatase at first. This is
lost steadily, however, beginning in the proximal levels, until at 19 days
the entire collar is negative to the phosphatase test (compare Fig. 3 with
Figs. 5 and 6).

In the ramogenous zone, most of the nuclei of the intermedium and
cylindricum show slight activity in early stages. By 18 days, however,
most of the nuclei have become phosphatase-negative. Reactive nuclei
remain in the ventral part of the feather for some time; they are confined
chiefly to the basal cells (Fig. 7). As barb ridges are formed from the
material in this ventral area, the nuclear activity is lost (see below).
The distribution of ribonucleic acid undergoes the greatest transformation in the development of the epidermis. The epidermis of the follicular wall maintains about the same concentration of RNA at its base; little remains in the distal portion. The collar and ramogenous zone accumulate more and more RNA in both the cylindricum and intermedium (compare Figs. 14 and 15, 16 and 17). These cells contain dark-staining nuclei and small, basophilic nucleoli. The nuclear stain is not due to RNA, but the nucleolar stain is, as proven by ribonuclease digestion. The cytoplasm contains a large quantity of RNA, much of which is located in the bases of the cells adjacent to the pulp. As the barb ridges begin to form in the ramogenous zone, these cells lose their elongate shape and conform to the shape of the ridge. The later fate of the RNA is discussed in the section on the differentiation of the barb ridges.

In the 16-day embryo, the middle layer of sheath cells has begun to split distally at the level of the surface skin. The split then proceeds basally until it reaches the collar. The thin outer layer unites with the epidermis of the follicular wall; the thick inner layer forms the sheath of the feather. At the collar region, these cells increase in number until they make up the greater proportion of the collar itself. Immediately peripheral to the inner layer of the epidermis, the sheath cells can first be distinguished by the contrast between their nuclei and those of the inner layer of cells. The nuclei of the sheath cells are round in comparison with the elongate nuclei of the inner cell layer, and contain large nucleoli which stain heavily with toluidine blue. The remainder of the nucleus, in contrast, shows little or no basophilia. The cytoplasm stains intensely with a basophilic substance that is lost after digestion.
with ribonuclease.

External to these inner sheath cells, the bodies of the peripheral sheath cells begin to flatten out. The nuclei appear much the same; however, the basophilic cytoplasm no longer fills the cells, but is confined to the periphery. Proceeding distally, the cells become progressively more flattened, and begin to lose their basophilia, until only the cell walls give a weak stain with toluidine blue. These walls become compressed to form the tough sheath which encompasses the feather.

The localization of the protein stain corresponds closely to that of the RNA; however, the two dyes do not reveal the same substance, since after treatment with ribonuclease, only the protein stain is retained. In the sheath cells, the protein is not lost as the cells flatten out, as is the case with RNA. Instead, the flattened cell walls stain heavily (Fig. 21), indicating the presence of large amounts of protein.

The blue tetrazolium test indicates a large number of disulfide linkages in the sheath and follicular cells. They stain darkly, starting just above the collar, and continue to react in the more distal areas of the sheath. The collar and inner layers of the feather do not stain, except for their nucleoli. The nucleolar reaction is particularly intense in the sheath cells within the collar itself.

As has been mentioned previously, PAS-reactive polysaccharides are not present in the epidermal cells at any time. However, the limiting membrane of the cells of the stratum cylindricum, which separates the pulp and epidermis, stains very intensely with the PAS reaction in the collar and ramogenous zone (see Fig. 2h). Most noticeable is the extreme irregularity of this membrane and of the internal boundaries of those cells adjacent to the pulp. On the pulp side, the membrane appears sharply
defined, but on the side lying against the epidermis it extends for variable distances between the cells. Above the collar, the membrane tends to flatten out, but is still very prominent. At the level of the ramogenous zone it grows thin at the inner side of the ridge, but thickens greatly as it passes with the cylindrical cells between the ridges. This could be explained by the mechanical stretching of the cylindrical cells around the barb ridges and their compression between these ridges. The final fate of this membrane will be discussed in the section dealing with the barb ridges.

Staining of frozen sections with Sudan black revealed that no lipids are present in the epidermis during the earliest stages of feather growth. As the collar becomes thickened into its definitive form at about 18 days, fine lipid granules appear in the cytoplasm of the inner and outer layers of cells, but not to any degree in the sheath cells. These granules are not entirely regular in size or position, but are all quite small and tend to be situated around the nucleus. These granules disappear in the ramogenous zone.

**Barb ridges**

The newly formed barb ridge consists of a peninsula of cells from the stratum intermedium bounded internally on three sides by the stratum cylindricum, which in turn is bounded by the limiting membrane and pulp. Peripherally, the sheath cells delimit the ridge. At about 15 days the cells of the barb ridges have a rather uniform appearance; they contain little phosphatase, but large amounts of cytoplasmic RNA. The PAS-reactive limiting membrane is thin at the inner end of the ridge, but thicker and strikingly reactive at its sides. The reactivity of this membrane has
decreased from the region of the collar (see above) and continues to do so as the ridges differentiate further, disappearing last from their lateral surfaces. The limiting membrane also contains a small amount of protein as revealed by the Hg-BPB test.

In feathers obtained from embryos of 16 days and older, the distribution of alkaline phosphatase differs from that of the other constituents tested in that it is not present in the barbule cells at any stage of their differentiation. There is a danger that diffusion phenomena may complicate the exact localization of the enzyme. However, the short incubation time (1 hour) and absence of diffusion into other areas adjacent to high concentrations of the enzyme have minimized this possibility. Alkaline phosphatase is absent from the barb ridges when they first form at 15 days, but appears shortly after the initial differentiation of the cells has begun. After the seventeenth day it appears in the limiting membrane and the nuclei of the stratum cylindricum, although not to any extent in their cytoplasm (Fig. 8). The activity is confined to the cells lying next to the pulp; the peripherally-located cells lying between the bases of the ridges are comparatively inactive. The maximal activity of the enzyme appears as the barbule cells of the intermedium begin to differentiate. Phosphatase activity then declines until, at the time the barbule is formed, the reaction is confined to the region of the cylindricum lying between the barb stem and the pulp. Even this disappears rapidly.

The remaining constituents for which tests were performed were related either in distribution or in time of appearance, and were concentrated in the barbule cells at various stages of their differentiation. They will be considered in the order of their appearance.
The alignment of the cells of the barb ridges has begun by 16 days. At the same time, there is a loss of cytoplasmic basophilia from all of the cells except the barbule plate cells of the intermedium. In these barbule cells, differentiation becomes apparent almost immediately. Glycogen appears in them at about 17 days. It arises in the form of very fine granules distributed throughout the cytoplasm of the cell. It appears first in the barbule cells next to the sheath, and later extends to the more centrally located cells. However, it does not reach the cells of the barb stem until much later. As the barbule cells elongate, more glycogen accumulates (Fig. 18) until the cells contain large amounts of it. The granules also tend to become somewhat coarser, although this is possibly a fixation artifact.

During the later stages of barbule formation, as cornification sets in, the glycogen disappears. Since this process begins in the peripheral cells, the glycogen is lost here first, and disappears later from the more centrally located barbule cells.

As the last of the glycogen is disappearing from the barbule cells, an accumulation of glycogen begins in the medullary cells of the barb stem. These cells have begun to enlarge at this time, and the increase of glycogen is rapid until the cells are filled with coarse granules (Fig. 19). The glycogen is lost slowly from the cells as their cornification becomes complete.

Shortly after glycogen begins to increase in the barbule cells, RNA also starts to accumulate. Unlike glycogen, it is not a new constituent of the cell, having been present even before the formation of the barb ridges, but as the barbule cells increase in size, the cytoplasm becomes
more basophilic (Figs. 22 and 23). This basophilia becomes increasingly apparent as the cells elongate (Fig. 20), partly due to actual increase in RNA and partly to a contrast with the cell nuclei. At this time the latter begin to enlarge and lose their basophilia, until they appear nearly colorless except for a large basophilic nucleolus. The RNA is lost from the barbule cells as they complete their differentiation. As far as can be seen, the medullary cells of the barb stem fail to acquire large amounts of RNA, although some is present.

Protein arises later than the previously mentioned substances. Although all of the cells stain with Hg-BPB, a definite increase in the barbule cells cannot be observed until they are quite elongated. This is at the time when RNA has reached a maximum, and the concentration of glycogen is declining. From this point on, the barbule cells give an intense protein reaction (Fig. 20). Correlated with their lower RNA content, the medullary barb cells do not show a high concentration of protein, although some is certainly present.

The same pattern can be seen even more clearly with the blue tetrazolium reaction for disulfides. In this case, the cells of the barb ridges show no reaction except for their nucleoli. These become very prominent in the barbule cells as the nuclei enlarge during the modification of the cell. As the cells become long and slender, they begin to show a blue-purple reaction which persists in the barbule and increases in intensity during the remainder of differentiation.

**Rhachis**

The rhachis is first visible in the wing feather of 16 days. At first it is only a broad region free from barbs, but as it differentiates,
it narrows progressively towards the apex and projects farther towards the center of the feather than the barbs. It is narrower next to the sheath and broader toward the pulp.

Unlike the barb ridges, the rhachis is comparatively free from alkaline phosphatase in all stages of its development. Its cells contain large amounts of cytoplasmic RNA, but without localization in any one area during most of development. As differentiation progresses, cells towards the inner edge of the rhachis begin to swell and appear to become vacuolated, much as the medullary barb stem cells of the barb ridges. They also accumulate a large amount of granular glycogen (Figs. 19 and 25), which persists for some time. This occurs somewhat above the level at which the cells of the barb stem acquire glycogen. Certain of the cells lying between these stem cells and the sheath are closely packed with large amounts of RNA, but it is absent in others (Fig. 20). Some of these cells swell and form a part of the medulla; the remainder condense about the medullary cells to form the cortex of the rhachis. The cortical cells contain much protein (Fig. 26), but the medullary cells show only a fine meshwork of protein granules and a heavy layer at the cell boundary.
DISCUSSION

With the foregoing observations on the juvenile feather in mind, it now becomes possible to compare the down and juvenile feathers with regard to their morphological and histochemical characteristics. It must be remembered, however, that these two structures cannot be considered completely independently of each other because of their common origin. For this reason, the differences between the two are not abrupt, but arise gradually.

Perhaps one of the most striking changes which occurs during the transition from the down to the juvenile feather is the morphological delimitation of the dermal papilla. A region corresponding to the papilla is present from the inception of the down feather, consisting of a condensation of dermal tissue beneath the epidermis as the latter rises above the level of the skin. Later it comes to lie at the base of the conical cylinder which makes up the down feather. As the feather then sinks into its follicle in the skin, this dermal condensation lies at its base, and gradually, as the juvenile feather begins to form, becomes distinct from the underlying dermis. The upper boundary of the papilla does not have actual structural limits, and is evident only in later stages, chiefly because of differential staining.

As the papilla develops, certain histochemical changes take place with relation to the base of the feather. The most noticeable of these is the upward shift of the lower boundary of phosphatase activity. In the down feather, this enzyme extends well below the base of the feather
and out into the surrounding mesenchyme, in diminishing gradient fashion, as would be expected in an embryonic field. However, as the follicle forms, phosphatase activity becomes limited to the dermis of the feather, with little activity in the connective tissue below it (cf. Fig. 2). During the development of the juvenile feather, this phosphatase-free area retreats still farther upward from its base, until the entire papilla is free of phosphatase. The rest of the pulp remains highly active over some period of time.

Following the same pattern as this shift in phosphatase is the progressive loss of acid mucopolysaccharide from the papilla except for a narrow peripheral region. In general, acid mucopolysaccharide is concentrated at the level at which phosphatase begins. It fades out distally rather quickly, however, in contrast to phosphatase, which remains active throughout the length of the pulp. This is comparable to the condition in the down feather, in which the localization of the acid mucopolysaccharide also closely corresponds to the sites of enzymatic activity at the base of the feather. An accumulation of glycogen also occurs just above the papilla. This is a new component of the dermis; it is not present in the down feather.

Within the papilla, there is a great increase of protein in the fibers and cells. This is foreshadowed in the down feather during its later development by a distinct increase in protein in the dermis at its base. PAS-reactive polysaccharides are present in the dermal papilla to a greater extent than in the down feather, but they are also present in small quantities in the down feather.

The manner of formation of the barb ridges is altered from that found
in the down feather (see p. 4). In spite of this, the walls of the epi-
dermal cylinders of the two show some similarity, particularly in the high 
RNA content of the innermost cells next to the pulp. In the juvenile 
feather this may be obscured by the large amount of RNA in the remaining 
cells, especially in the sheath, but the high accumulation of RNA in the 
cytoplasm of the basal cells continues. This means that, in both types of 
feathers, substances passing into the epidermis from the pulp first en-
counter the RNA.

The histological differentiation of the barbs and barbules is essen-
tially the same in the down and juvenile feathers. Glycogen is found in 
the barb stem along with smaller amounts of RNA and protein. Alkaline 
phosphatase can be seen in the membrane bounding the ridge, and frequently 
in the adjacent innermost layer of cells of the barb ridge itself. The 
further specialization of the barbule plate within the ridge, showing 
successive changes in glycogen, RNA, and protein content, is not shown as 
clearly in the down as in the juvenile feather. Glycogen was not found 
at all in the barbule cells of the down feather. From these modifications 
it might appear that the rates of growth and differentiation are not the 
same in both types of feathers.

With the formation of the juvenile feather, the rhachis develops 
dorsally, thus changing the feather from a radially to a bilaterally 
symmetrical structure. Its lateral surfaces arise from the fused bases 
of the barb ridges, but its central portion is derived from cells of the 
collar. It bears a close resemblance to the barb stem in certain respects, 
especially in the morphological and histochemical development of the 
medullary stem and its surrounding cortex.
With these relationships in mind, it is now possible to turn to the problem of the significance of these various components and their relationship to one another and to the development of the feather.

Alkaline phosphatase is a common constituent of many tissues. It is present, for example, in large amounts in cartilage at the time of calcification (Moog, 1946), in the proximal tubules of the kidney, the brush border of the intestine (Davidson, 1947), in healing epidermal wounds (Fell and Danielli, 1943), and in such developing embryonic structures as the papilla of the hair (Bourne, 1943; Johnson et al., 1945), and the feather (Johnson and Bevelander, 1947a). Because of its wide distribution it is reasonable to suspect the presence of more than one enzyme, and there is some evidence that this is the case (Newman, Feigin, Wolf, and Kabat, 1950). The theories concerning the function of the enzyme have been discussed in a previous study (Koning, 1953), and only some of the pertinent propositions will be considered here.

One suggestion has been made that alkaline phosphatase acts as a phosphorylating agent in glycogenesis (Wislocki and Dempsey, 1945; Johnson and Bevelander, 1946). The enzyme is often related to glycogen deposition, as in the placenta, bone, and the epidermis of pig embryos; but the inability to correlate many major sites of phosphatase activity to glycogenesis has made this theory somewhat untenable for general application.

Phosphatase may be involved in the formation of structural proteins. Fell and Danielli (1943) proposed this theory on the basis of studies which showed a large amount of phosphatase on the collagen of healing epithelial wounds in the rat. This has been supported by other studies on such divergent structures as the uterus (Atkinson and Elftman, 1947;
Rings, 1950), where estrogens produced an increase in phosphatase in the epithelial wall, and on the silk and salivary glands of insects (Bradfield, 1951b; Lesher, 1952), which contain phosphatase in their epithelium during actively secreting stages. Bradfield further suggested tentatively that phosphatase might liberate the proteins from a nucleic acid complex after their formation.

On the other hand, Robertson, Dunihue, and Novikoff (1950) found that fibrous tissue could be formed without phosphatase in some instances. Cold and Gould (1951) reported that the presence of phosphatase on collagenous fibers may not be due to activity of the enzyme at that site, but to adsorption of the enzyme. Bern and Levy (1952), in studying the phosphatase activity of the genital tract of male rats under various conditions, concluded that there was no consistent correlation between phosphatase and synthesis of fibrous proteins. For these reasons, the connection between phosphatase and synthesis of protein is somewhat tentative.

Moog (1944) found a suggestive parallel between the presence of phosphatase and chemodifferentiation. Phosphatase activity seemed to be related to a high concentration of nucleic acids (Moog, 1946). Johnson and Bevelander found that the localization of phosphatase in the developing hoof of the pig (1947b), and in the developing feather (1947a) suggested a chemodifferentiating function for phosphatase. This was also true of the findings of Koning and Hamilton (1954) on the down feather.

Danielli (1951, 1953) suggested that this enzyme acts as a phosphokinase in tissues. In this role, phosphatase would break high-energy
phosphate bonds to cause the contraction of proteins within the cell membrane, thus bringing a variety of molecular substances into the cell.

In the developing down feather, the early appearance of large amounts of dermal phosphatase in and below the feather suggested that phosphatase might have an inductive function in the early stages of development and differentiation of the feather. In the juvenile feather, phosphatase is conspicuously absent from the dermal papilla after it is fully formed. The localization of phosphatase in early stages of the formation of the feather might indicate a relationship to the initial induction and organization of the feather. This would be substantiated if the early development of the regenerating feather were found to contain phosphatase.

From the fact that phosphatase is not present in the dermal papilla of the juvenile feather during the time that it is forming pulp cells, it can be inferred that the enzyme is not primarily concerned with cellular multiplication. In the pulp exclusive of the papilla, the high concentration of phosphatase may play an important part in the basic functioning of the pulp, which, according to Lillie (1940), includes supplying nutrients to the epidermal cylinder and supporting the tissues of the epidermis prior to cornification.

There are several possible ways in which phosphatase might be involved in supplying nutriment to the epidermis. It might act solely as a transport mechanism; perhaps its action as a phosphokinase supplies the energy needed for such a transport. In addition, it might act as a selective system, both in the pulp and basement membrane of the epidermis, preferentially transporting only certain substances (see below). Phosphatase
could also modify the transported substance, for example, by phosphorylating glucose, which would allow it to be utilized more rapidly in the cells of the barb ridges.

As a means of providing support, the enzyme would appear to function chiefly in the differentiation of the elements of the pulp itself. The cells of the pulp could utilize the phosphatase in synthesizing the materials needed for them to attain their definitive form. Further, phosphatase might have a part in the forming of collagenous fibers and ground substance by the cells of the pulp. Thus, the activity of phosphatase might supply energy for intercellular transport, the synthesis of protein, and chemodifferentiation.

The low grade activity of phosphatase in the collar and follicular walls, as well as the ventral region of the ramogenous zone, may be an artifact caused by diffusion of reaction products from the pulp (see Gomori, 1952, and Danielli, 1953, for a discussion of this problem), although no evidence of diffusion was found in other cells adjacent to regions of high activity. The nuclear position of the enzyme in these latter places seems to relate it to early stages in the differentiation of the cells during the formation of the collar. It soon disappears from the collar, but persists in the ramogenous zone ventrally, which may reflect a changed status of the cells of the collar. Phosphatase is clearly not needed in later stages of the differentiation of the barbule cells, because they are totally unreactive.

In summarizing the above possibilities, it would seem from the comparative localization of phosphatase in the down and juvenile feathers,
that phosphatase has a dual function as suggested by Moog (1946). The initial presence of phosphatase in the down feather and its occurrence in the epidermis of the collar and ramogenous zone of the young juvenile feather would be connected with the chemodifferentiation of these tissues. After differentiation had occurred, the phosphatase that was still found in the pulp would serve in the functional activity of the pulp. In the latter case phosphatase would help to transport materials from the pulp to the epidermis. This possibility is supported by the fact that at the level at which barb ridges are undergoing rapid differentiation, phosphatase-active pulp is closely applied to the inner edges of the barb ridges, and the basal membrane and stratum cylindricum reflect some of this activity. This would be expected if its chief function were to pass raw materials across the membrane to the cells of the epidermis.

If any comparisons can be drawn between the phosphatase of feathers and that of hair, it would seem that the idea of a transporting function could account for most of the activity in the hair. No pulp is formed, but the papilla is phosphatase-reactive during the entire period of growth of the hair. It would provide a means of rapid transmission and modification of materials for the construction of the hair shaft.

Fewer functions have been ascribed to ribonucleic acid within cells than to phosphatase. Claude (1948, 1950) proposed that RNA is connected in some way with the ability of a cell to carry on anaerobic glycolysis, but this idea has received little support. Lansing and Rosenthal (1952), on the basis of experiments with Arbacia eggs, postulated that the initial step in the transfer of material from the external milieu to the interior
of the cell involves the formation of a complex between the external material and the RNA at the cellular surface. While this theory would apply satisfactorily to the RNA in the cylindrical cells abutting the pulp, which might act as an acceptor for substances passing into the epidermal cells from the pulp, it does not explain clearly the high concentration of RNA in the barbule cells. The localization of RNA in all the cells is perhaps best explained by the theory which connects RNA with the synthesis of protein (Caspersson, 1947; Brachet, 1947). Although its mechanism of action is not known, Brachet suggested that RNA might function as a protein precipitant, facilitating further synthesis.

The amount of RNA in the collar cells is such that it indicates a high activity of the cells. In the inner zone, RNA is probably concerned mainly with the increase in cytoplasm, because the cells here are undergoing rapid mitosis. The RNA of the sheath and barbule cells cannot be so involved, since these cells are no longer dividing. In these regions RNA is probably concerned with the production of keratin or its precursors, as indicated by the great increase in proteins in these regions (see below).

The work of Giroud and Bailliard (1930) on the areas of keratinization in the feather is substantiated by the present studies. In the sheath and follicle, protein appears just above the collar, but the first signs of keratinization in the barb ridges occur in the formation of the barbules, and then in the barb stem. Both of these occur at a higher level in the feather.

In connection with the formation of protein, it can be seen that in each case RNA precedes rapid keratinization. RNA appears in the sheath cells in the collar; protein begins to accumulate just above the collar.
In the barb ridges, RNA precedes the keratinization of the barbule cells by a short time. Thus it appears that RNA must be present within the cell at the time of keratinization. Whether RNA actually forms the protein, or whether its presence is required at the time of keratinization to enable the protein molecule to attain its final shape after it has been formed cannot be decided on the basis of the present work.

The appearance of the nucleus reflects some of the changes which take place during keratinization. In the cells of the intermedium, the nuclei are basophilic, with small nucleoli. As the barbule cells start to differentiate, the nucleus loses its basophilia, chiefly due to a loss of DNA. The nucleolus, on the other hand, becomes large and heavily charged with RNA. This is accompanied by a build-up of RNA in the cytoplasm and a parallel rise in the concentration of proteins. Towards the end of keratinization, the nucleus becomes indistinguishable within the cornified cell.

The close correspondence of metachromatic acid mucopolysaccharide and alkaline phosphatase in both the down and juvenile feathers appears to be more than coincidental. This substance is usually a sulfate-containing mucopolysaccharide, closely related or identical to substances in the dermis of the skin which provide support. The mucopolysaccharide molecule may be structurally associated with that of the phosphatase, or it may have a nutritive function (Bunting, 1950) along with the enzyme. Similar inferences also apply to the papilla of the growing hair. Although the papilla remains small and needs little support, the nutritional requirements of the epidermal hair may be met in part through the activity of the mucopolysaccharide.

Moog and Wenger (1952) associated a PAS-reactive polysaccharide with sites of phosphatase activity in a series of organs including the feather.
not as a filler, permitting preferential passage of certain materials needed
the orientation of substances in the path that pass into the path. Further, the
membrane may be important in
become phosphatase-positive. The junctional membrane becomes less reactive around the base of cells, but here it
epithelial is simplified with the high concentration of cytoplasmic RNA
which separates the cytoplasm-epithelium, phosphatase-actin part from the
in the membrane, it may consist of a protein-actinocyturic complex,
Pi-g-actin-actinogen and the presence of small amounts of protein
cells of the epithelium. In view of the structural naure of many of these
it is of a tubular nature. The cell membranes that were the other
fact that a barrier is still present between the dermis and the pulp
rather than a true basement membrane, however, the presence of the
be composed of the inner cell layers and junctional complexes of the stratum corneum
this PAS-actin-actinogen appears to
membrane that is present that does not seem to be a basement membrane
put in the connective tissue, some tissue of interest. Figure (1940)
the PAS-actin-actinogen of the internal junction membrane bounding the
function cannot here be directly correlated with phosphatase activity
with the idea of a supportive function in the dermal tissue, but such a
correlated. The latest extracellular location of the material correlates
contrasted to the pulp, would indicate that the two are not necessarily
have the presence in the pulp and collagen demarcate with phosphatase
the extracellular. The PAS-actin-actinogen in the connective tissue is located
They postulated that it might have a supportive and orientation function for

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at this level. The change in the reactivity of this membrane at higher levels would then reflect a corresponding change in permeability to substances.

Glycogen accumulates in the barb ridges during the early period of their differentiation. It tends to be deposited in active regions that have only a limited blood supply (Wislocki and Dempsey, 1945; Bradfield, 1951a), and is utilized subsequently in anaerobic metabolism. This would explain the earliest deposition of glycogen at the outer edges of the barb ridges, which are farthest from the nutrient supply in the pulp, and its later spread towards the inner edges.

From the presence of glycogen in the barb ridges and hair, it might appear that glycogen is necessary for the process of keratinization. If this is so, then its absence in the sheath is somewhat difficult to explain. Montagna, Chase, and Lobitz (1952) suggest that glycogen is deposited at sites where the damming of keratinized material has retarded the normal rate of keratinization. If true, the delay in keratinization of the barbule cells might account for their accumulation of glycogen. This would also be the case in the medullary cells of the barb stem. The glycogen would then be utilized when keratinization of the cells finally proceeds. Presumably, such a block in keratinization does not occur in the sheath cells, since glycogen does not accumulate in them.

With regard to the presence of glycogen and its relation to keratinization, it is of interest to note the time sequence of glycogen deposition in the barb ridges. Since it appears first in the barbule cells, and disappears from them before it is found in the medullary cells, it would
seem that the former are keratinized before the latter. Further, glycogen appears in the medullary cells of the rhachis after it is first found in the barb stem cells, so that the rhachis would appear to be keratinized last. The presence of glycogen in these medullary cells for some period of time suggests a delay in the keratinization of these structures, which agrees well with their lower content of RNA and protein at the time the barbule cells are keratinizing.

The glycogen in the pulp just above the papilla seems paradoxical in view of the fact that the pulp is supposedly well supplied with blood vessels. It must be remembered, however, that just above the papilla, the arterial system is poorly developed (Lillie, 1940), since pulp is continually being formed in this region, and the blood vessels attain their fully differentiated form only at more distal levels. This, combined with the rapid differentiation of the pulp cells just after they leave the region of the papilla, brings about the same conditions noted previously as prerequisite for glycogen deposition, viz., active cells in a region that is relatively poorly nourished.

The presence of sudanophilic granules in the cells of the collar indicates the presence of lipids or phospholipids. From the nature of the granules and their position around the nucleus, it is possible that they represent the Golgi substance. If this is the case, these cells in the collar must be physiologically different from the other cells of the epidermis. In the pulp, the lipid-containing cells are mostly macrophages, such as found by Wislocki and Dempsey (1946) in the connective tissue of rats.
SUMMARY AND CONCLUSIONS

1. The developing juvenile flight feather of the White Leghorn chick embryo was studied by histochemical procedures in order to identify the positions of some of its more important constituents from the time of its organization at 13 days until the second day after hatching, at which time the rhachis was well formed and the distal epidermal components had attained their final differentiated state. The significance of these substances was discussed with reference to their localization in the down feather and hair.

2. Alkaline phosphatase became progressively limited to higher levels of the feather. In the down feather it appeared in and below the pulp in a pattern typical of a gradient field. As the juvenile feather developed, there was a progressive loss of the enzyme from the base of the feather concurrent with the development of the dermal papilla. At the time this structure was clearly defined, phosphatase was conspicuously absent from it. Epidermal phosphatase was present in the nuclei of the collar in early stages of its development and in the ramogenous zone on the ventral side of the feather. In the barb ridges, phosphatase was present in the basement membrane and stratum cylindricum during the most active period of barbule differentiation, resembling the down feather in this respect. It was suggested that in the development of the down and juvenile feather, phosphatase plays a dual role; first in the chemical differentiation of cells in the down feather, and second in the transport and/or modification of raw materials passing into the epidermis in actively differentiating regions.
3. As in the down feather, ribonucleic acid was found chiefly in the epidermis. In the sheath cells it was very concentrated in and above the collar, fading out distally. In the cylindricum and intermedium, all of the cells contained considerable accumulations of RNA, especially in the cytoplasm that lay adjacent to the pulp, much as was the case in the down feather. After the formation of the barb ridges, the RNA was lost from all of the cells except those making up the barbule plate. Here it continued to accumulate until the barbule was nearly completed, after which it dwindled away. Because of the close correlation between RNA and sites of large quantities of proteins, it was suggested that RNA was in some way related to the synthesis of proteins.

4. Paralleling ribonucleic acid, proteins were found in large quantity in the sheath above the collar and in the barbule plate cells in later stages of their formation. In addition, massive concentrations in the fibers and cells of the papilla were observed in later stages of development. Disulfide tests indicated that these linkages were present in the sheath and barbules, but not in the papilla.

5. Polysaccharides reactive to at least two staining techniques were observed. Metachromatic acid mucopolysaccharides were abundant in the pulp just above the papilla. PAS-reactive polysaccharides were present in the papilla as well as the pulp, and also in the dermal tissue of the follicle. In addition, a PAS-reactive basal membrane was present between the pulp and stratum cylindricum in the collar and ramogenous zone. Acid mucopolysaccharide seemed to be involved in support and transport of nutritive substances to the epidermis; whether it was connected with
phosphatase or not was not determined. PAS-reactive polysaccharides seemed to serve in the support of the pulp and feather cylinder.

6. Glycogen was present in the pulp just above the papilla, in the medullary cells of the barb stem and of the rhachis, and in the barbule cells during nearly all of the early part of their differentiation. Glycogen deposition seemed to be correlated with keratinization; it also might be utilized by active cells having a poor blood supply.

7. Lipids were confined to fine granules in the cytoplasm of the collar cells and to large coarse granules in macrophages of distal portions of the pulp.
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FIGURES 1-9 WERE ALL INCUBATED FOR ONE HOUR AT 37° C., IN A SOLUTION CONTAINING GLYCEROSEPHOSPHATE, THEN TREATED TO REVEAL PHOSPHATASE.

FIG. 1. Longitudinal section of a down feather from an 11-day embryo showing the concentration of alkaline phosphatase at and below the base of the feather. Note the spreading of the reaction into the mesenchyme beneath the base and the linking of reactive areas of adjacent germ. X 90.

FIG. 2. Longitudinal section of a juvenile feather from the wing of a 15-day embryo. Note the reactivity of the pulp of the feather spreading slightly below the base at the periphery of the feather cylinder. X 90.

FIG. 3. Longitudinal section of a wing feather from a 16-day embryo, showing the dermal base of the feather free from phosphatase except next to the epidermal cylinder, where activity extends nearly to the level of the collar. The nuclei of the collar and ramogenous zone just above it are also somewhat reactive. X 90.

FIG. 4. Longitudinal section of a feather from a 17-day embryo. There is some phosphatase next to the epidermis, extending towards the base of the pulp. Activity can be observed in the nuclei of the collar and ramogenous zone, and also in the follicular cells. The activity in the follicle appears as a dark line outside the feather proper. X 35.

FIG. 5. Longitudinal section of a feather from the wing of a 19-day embryo. All activity has disappeared from the base of the feather. Phosphatase-active pulp extends between the barb ridges. The axial artery can be seen in the center of the pulp. The feather has enlarged considerably from the size seen at seventeen days (Fig. 4). X 35.

FIG. 6. Longitudinal section of the lower part of a wing feather after hatching. The axial artery passes up the center of the pulp. Blood sinuses can be observed lateral to it. Note the abrupt start of phosphatase activity in the center of the feather, with narrower peripheral loci of activity. These two regions merge distally to form the cylinder of active pulp.

FIG. 7. Cross section of the juvenile wing feather of a 20-day embryo at the level of the ramogenous zone. The central, highly reactive pulp can be seen, surrounded by less reactive pulp which is closely applied to the epidermis. The spaces near the dermo-epidermal boundary are probably artifacts. Activity can also be observed in the nuclei of the stratum cylindricum. X 300.
Fig. 8. Cross section of a 22-day wing feather at the level of barb formation. Note the phosphatase-reactive pulp closely applied to the ridges and the activity of the basement membrane and nuclei of the stratum cylindricum. Phosphatase-active pulp adheres closely to the rhachis (right) also, but the epidermis is non-reactive.

Fig. 9. Tangential section of the barb ridges from a 19-day embryo. Phosphatase-reactive pulp passes up between the barb ridges for some distance.
Figures 10-17 are longitudinal sections of juvenile wing feathers of chick embryos taken at various stages of development.

Fig. 10. Feather from a 17-day embryo treated with the periodic acid-Schiff's reagent. The pulp stains weakly. The coarsely granular substance in the pulp above the collar level is glycogen, which is beginning to accumulate at this stage. X 100.

Fig. 11. Feather from a 22-day embryo stained by the PAS technique. Note the dark granules of glycogen situated above the papilla. The remainder of the pulp and papilla stains weakly. The epidermis is entirely negative. X 35.

Fig. 12. Wing feather from a 15-day embryo stained for protein with mercuric chloride-bromphenol blue. The pulp and epidermis both stain. In the base of the papilla the heavier stain indicates the early accumulation of fibers. The dark, branched structures in the epidermis are melanophores. X 100.

Fig. 13. Wing feather from a 22-day embryo stained for protein with Hg-BPB. Note the heavy stain in the epidermis, and especially the deeply stained papilla, which consists of closely packed cells and heavily-staining fibers. X 35.

Fig. 14. Wing feather from a 16-day embryo stained with toluidine blue. The pulp adjacent to the epidermis in the collar and ramogenous zone stains more than the central areas. The epidermis and follicular wall also show considerable basophilia. X 100.

Fig. 15. Wing feather from a 16-day embryo stained with toluidine blue after digestion with ribonuclease. There is loss of cytoplasmic basophilia that is particularly apparent in the sheath cells and in the narrow ridge of epidermal cytoplasm bounding the pulp. X 100.

Fig. 16. Wing feather from a 22-day embryo stained with toluidine blue. The great accumulation of RNA in the epidermis can be seen. On the left is a lighter area in which RNA occurs only in the peripheral sheath cells. The follicular wall also stains heavily. X 35.

Fig. 17. Wing feather from a 22-day embryo stained with toluidine blue after digestion with ribonuclease. The basophilia of the cytoplasm has disappeared. The sheath nuclei are colorless in contrast to the dark nuclei of the cylindricum and intermedium. The dark stain in the pulp above the papilla is the metachromasia of acid mucopolysaccharide. X 35.
 FIGURES 18-21 are all cross sections of juvenile wing feathers at the level of the barb ridges. X 300.

Fig. 18. Feather from a 22-day chick (2 days post hatching) stained by the PAS technique. The follicle around the feather stains darkly due to an unidentified polysaccharide. In the barbule plate, glycogen stains in the cytoplasm of the more peripheral cells. It is especially concentrated at their inner edges, which are elongating toward the sheath at this level.

Fig. 19. Feather from a 22-day chick (2 days post hatching) stained for glycogen. Note the large granules in the medullary cells of the barb stems and rhachis (center). This is at a more distal level than Fig. 18, and the barbule cells are fully differentiated (top) and do not stain.

Fig. 20. Feather from a 22-day chick (2 days post hatching) stained with toluidine blue to demonstrate RNA. The inner ends of the barbule cells stain darkly in contrast to the large, pale nuclei with their prominent, darkly stained nucleoli. At the top is the rhachis, showing the irregular localization of RNA in the cells near the sheath (left). The undifferentiated cells at the inner edge of the barb ridges next to the pulp will form the medulla and cortex of the barb stem.

Fig. 21. Feather from an 18-day embryo stained with Hg-BPB for protein. The sheath stains heavily (left). The barbule cells are attaining their fully differentiated state and contain considerable protein. The large medullary cells of the barb stem fail to show much protein.
Fig. 22. A tangential section of a feather from a 19-day embryo showing RNA in the barb ridges. The barbule cells contain considerable cytoplasmic RNA (arrow). X 430.

Fig. 23. A similar feather stained with toluidine blue after digestion with ribonuclease. Note the loss of basophilic staining in the barbule cells (arrow). X 430.

Fig. 24. An oil immersion photograph of the collar region of a feather (22-day embryo) after staining by the PAS method. Note the heavy stain in the follicular dermis (top). The sheath has not yet split into two layers at this level. At the inner edge of the epidermis an irregular membrane can be seen between this layer and the papilla. The papilla (bottom) also contains a PAS-reactive polysaccharide. The stained material shown in these regions is not glycogen.

Fig. 25. A longitudinal section of the rhachis of a feather (22-days; i.e., 1-day post hatching) stained with PAS to reveal glycogen. Note the irregular size and distribution of the granules in the medullary cells. X 430.

Fig. 26. A longitudinal section of the rhachis of a feather (22-days) stained for protein by Hg-BPF. Note the heavy stain of the inner cortical layer (right) and the dark-staining cell walls of the medulla. The nuclei are prominent, but little cytoplasm can be seen. X 430.