Development of integumentary derivatives in the chick embryo as studied by histochemical and grafting techniques

Joan Leigh Thomson

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DEVELOPMENT OF INTEGUMENTARY DERIVATIVES IN THE CHICK EMBRYO
AS STUDIED BY HISTOCHEMICAL AND GRAFTING TECHNIQUES

by

Joan Leigh Thomson

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

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Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa
1963
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INTRODUCTION

The question of the origin of inductive specificity has intrigued embryologists for many years, but it is only in recent times that this perplexing problem has been elucidated with respect to specific embryonic systems.

The developing system with which this study is concerned, the integument and its derivatives in the chick embryo, provides a good example of inductive specificity. Interactions between the two layers of the integument, the dermis and the epidermis, result in the calling forth of developmental activity in the epidermis to form regionally specific structures. Many recent studies have been concerned with the relationship between the inductor, the dermis, and the reactor, the epidermis. Other investigations have been carried out on the histochemistry of the development of the feather, the most common of the integumentary derivatives.

Although it is generally believed that the dermis is the initial inductor of specific integumentary derivatives, the basis and origin of dermal inductive specificity have not been determined. In some recent investigations certain results have suggested that the deeper tissues below the dermis may be involved.

The problem which is considered in this investigation is the origin of and basis for dermal specificity, as studied by two different methods.

In one set of experiments chorio-allantoic grafts were made of combinations of skin and deep tissues from different areas of the embryo and at various stages of development. For example, skin from a
prospective feathered area was grafted to deep tissue of a prospective scaled area from which the skin had been mechanically removed; the combination was then grown as a chorio-allantoic graft. Conversely, skin from a prospective scaled area was grafted to deep tissue from a prospective feathered area. The purpose of these experiments was to see if the deeper tissue would have any influence on the specificity of the dermis, and thus on the developing integumentary derivatives. If so, this would suggest that the deep tissue provided the dermis with an influence, chemical or otherwise, which endowed the dermis with an inductive specificity. If the deep tissue had no effect on the outcome of the induction, this would suggest an innate specificity of the dermis.

In the second part of the study, histochemical stains were applied to sections of scale-forming tissue, at various stages of development, in order to locate certain enzymes, proteins, polysaccharides, and nucleic acids. A similar study, previously carried out on the developing down feather of the chick (Koning, 1953), was used as a guide and as a basis for comparison with the present study of scales. It was expected that the same substances would play a role in the development of both types of derivatives. It was hoped, however, that the histochemical tests might provide a biochemical basis for distinguishing between scale- and feather-forming tissue, because of the quantity, localization and/or time of action of the various chemical constituents of the developing organs.

The following report presents the results of these studies, and their interpretation in view of the pertinent literature.
PART I: GRAFTING
REVIEW OF LITERATURE

Most of the literature concerned with the development of the limb bud and the integumentary derivatives involves either the interactions of undifferentiated mesoderm and the overlying ectoderm, or those of the differentiated dermis and epidermis in the process of formation of derivatives.

One of the early studies on ectoderm-mesoderm relationships in the early chick wing bud was carried out by Saunders (1948), who found that wing materials were laid down in a proximo-distal sequence by the apical mesoderm, and that excision of the apical thickened ridge of ectoderm suppressed the process of development.

Zwilling (1955, 1956a) transplanted ectoderm from one 3-day limb bud to the mesodermal core of another 3-day limb bud in order to study the relationship between the two germ layers. In wing-leg exchanges the apical ectodermal ridge was necessary for an outgrowth to occur, but the mesoderm determined the nature of the outgrowth. Transplanting ectoderm from different regions of the donor limb showed that only the apical ridge was capable of bringing about limb development. On the other hand there appeared to be a factor in the mesoderm, concentrated in the central region of the limb-bud, which was necessary for the maintenance of the apical ridge.

The role of the apical ectodermal ridge as a significant morphogenetic factor was questioned by Amprino and Camosso (1959a, 1961), who believe that the ectoderm serves only to protect the mesoderm from the embryonic environment. Other extensive observations and experiments strongly
indicated that the apical ridge does have a morphogenetic role (Bell et al., 1959b, 1962; Zwilling et al., 1960; Gasseling and Saunders, 1961).

In more detailed studies of ectoderm-mesoderm interactions, Zwilling further elucidated the roles played by the two germ layers. He found (1956b) that the ectoderm was involved in the establishment of the axis of the limb, since rotation of the ectodermal ridge 90° caused the grafted limb to grow at right angles to the original orientation of the mesoderm. By supplying the mesoderm from one limb bud with two apical ridges, Zwilling (1956c) was able to bring about duplication of the limb, indicating a rather widespread capacity for response in the mesoderm. Zwilling and Hansborough (1956) studied the factors causing excess digitation by exchanging ectoderm and mesoderm between genetically polydactylous and normal limb buds from 3-day chicks. They found that the polydactylous condition occurred mainly in the presence of mesoderm of genetically polydactylous type, and proposed that the mesodermal maintenance factor might be more diffuse in the mutant.

Saunders et al. (1958) and Saunders and Gasseling (1959) found that severing and reversing the apical zone of a limb bud (mesoderm plus apical ridge) caused duplication of distal wing parts in most cases, probably due to the more widespread distribution of the mesodermal maintenance factor as a result of the reversal.

These studies indicate that the two layers of the developing limb bud represent a complex and highly integrated embryonic system.

Extensive studies on the interactions of ectoderm and mesoderm in relation to the development of integumentary derivatives were made in
Saunders' laboratory, by transferring blocks of mesoderm from the 3- or 4-day leg bud into 3- or 4-day wing buds from which similar blocks of tissue had been removed. In the first experiment of this nature, Cairns and Saunders (1954) found that thigh mesoderm implanted in the prospective shoulder region resulted in the development of feathers of thigh type in the shoulder tract, while apical leg mesoderm placed in the tip of the wing bud resulted in wings terminating in large foot-like structures. These results suggested that leg mesoderm could direct the course of development of wing-bud ectoderm, although exchanges between Silky hosts and Leghorn donors showed that the ectoderm determined the genetic type of derivative formed. Later investigations (Saunders et al. 1956, 1957a) showed that thigh mesoderm transplanted into the apex of the wing resulted in the formation of foot parts from host ectoderm, while apical leg mesoderm placed in the shoulder region resulted in the development of thigh feathers. Older apical leg mesoderm (4½ days) was able to induce foot parts in the shoulder region. Thus the morphological result of such exchanges appeared to depend on three interrelated factors: the source of the graft mesoderm, the location of the graft in the wing bud, and the age of the donor. Saunders et al. (1957b) studied the role of the germ layers in the origin of pattern in feather tracts by rotating blocks of tissue in the humeral tract 180°. The resulting abnormalities led Saunders to conclude that organization of the tract was accompanied by a progressive restriction of developmental potencies of the mesoderm of the tract.

The general conclusions from these studies by Saunders and his
collaborators were: (1) the mesoderm shows differences in inductive specificity exercised by closely associated regions, which may be related to underlying skeletal parts, (2) the mesoderm gives specific inductive information to the overlying ectoderm, subject to genetic limitations, (3) the apical ridge is able to modify the inductive specificity of the mesoderm. The exact time of the inductive action and the source of the inductive specificities in the mesoderm are unknown, but it is apparent that regionally specific epidermal derivatives arise from a "complex inductive interplay between their epidermal and mesodermal components during early embryonic development" (Saunders 1958). A diagram presented by Saunders (1958) illustrates well the interplay of various factors (see Diagram 1).

Similar results were obtained with limb buds of the chick by Hampé (1959), who found that, while the apical ectodermal ridge was necessary for outgrowth, it was the mesoderm which determined the course of development.

In another type of experiment, Zwilling (1959) studied combinations of tissues from ducks and chicks. He found that duck mesoderm could induce webbing in chick ectoderm, while chick mesoderm did not prevent webbing in duck ectoderm; thus, the ectoderm seemed to play more than a passive role in development.

Further support for these hypotheses came from the work of Kieny (1960), who grew naked mesoderm from a 3-day limb bud as a flank graft. In many cases the host epidermis covered the graft and was induced by the mesoderm to form an apical ridge. The ridge caused the outgrowth of a
supernumerary limb, with epidermal derivatives which corresponded to the donor mesoderm.

From these numerous studies of the relationships between the undifferentiated mesoderm of the early chick limb bud and the overlying ectoderm, it can be seen that in all cases the mesoderm is the primary inductor, although the ectoderm may influence the induction.

Many experiments have been devoted to the study of interactions between the differentiated dermal layer and the overlying epidermis. Sengel (1956, 1957a) removed skin from the middorsal region of the chick,
separated the two components and grew various combinations in culture for five days. Skin layers of three stages were used: an indifferent stage (less than 6½ days), a stage of mesodermal condensations (6½ to 7-1/4 days), and a stage of young feather germs (more than 7-1/4 days). It was found that dermis having mesodermal condensations could induce feathers to form in indifferent epidermis, while indifferent dermis could not. By the stage of young feather germs, the dermis was no longer able to call forth feathers from indifferent epidermis, which indicated a fairly short period of dermal induction. The epidermis from the stage of young feather germs, however, was able to form feathers with indifferent dermis, indicating that epidermis which had begun to differentiate was capable of organizing the dermis. Thus, development of the feather occurs in three phases: a stage of mesodermal condensations, of which the inductor is unknown, a stage of dermal induction, and a stage of epidermal induction.

In studying the question of the origin of the dermal condensations, Sengel (1957b) found that tissues from many organs (neural tube, liver, lungs, kidney, and muscles) were able to induce feathers to form in indifferent skin, suggesting a relatively non-specific chemical role for the substrate. In a later experiment Sengel (1960) succeeded in evoking feather germs in cultured skin using a water-soluble brain extract.

In further combinations Sengel (1958) found that back dermis, containing dermal condensations, combined with leg epidermis, which had already begun to form scales, resulted in the formation of feathers. In the reverse combination, scale-forming dermis with condensations induced
differentiating feather epidermis to form scales.

Rawles (1960-61) combined various stages of dermis and epidermis and grew them on the chorio-allantoic membrane. She found that when leg dermis in the process of scale formation (9-15 days) was combined with back epidermis in the process of feather formation (5-9 days), leg dermis younger than 13 days formed feathers, while leg dermis older than 13 days formed scales and/or abortive feathers. In the reverse combination, back dermis (6-8 days) could induce leg epidermis (9-13 days) to form feathers in all cases. It was concluded that either the leg dermis was a weak inductor, or it reached full strength only after 13 days of development. It was also suggested that the leg skin might be bipotential in character, being able to form both scales and feathers under certain conditions.

These experiments all indicate that the primary inductor of epidermal derivatives is the dermis. The source of the dermal inductive power and the time at which specificity is first established are not known.

Studies have been made in recent years of the nature of the interaction between dermis and epidermis. McLoughlin (1961) found that normal keratinization occurred in isolated 5-day chick epidermis only when the epidermis was replanted on the limb mesenchyme. Varying degrees of abnormal keratinization occurred when the epidermis was placed on gizzard, proventriculus, or heart mesenchyme.

Wessells (1961b) combined isolated 10- to 11-day leg epidermis with dermis of the same age in a chemically defined medium. Where a junction
was established, normal epidermal development ensued, although scales were no longer formed. Such tissue interactions were able to occur through a millipore filter, indicating that epidermal differentiation and survival depended on the proximity, but not the direct contact, of epidermal and mesodermal tissue. These experiments also showed that morphogenesis of scales and normal histological differentiation are not inextricably related.

Bonetti (1959) tested the hypothesis that dermis is the primary inductor by substituting chorionic epithelium for the epidermis. After several days in culture, the epithelium developed several layers of cells, indicating that the dermis was able to induce the differentiation of epidermis in the chorion, although no scales or feathers were formed.

A series of experiments performed by Amprino and Camosso (1959b) involved the experimental production of feathers in embryonic skin in which scales were differentiating. When the distal one-third of a chick leg bud (stages 21-24) was grafted either to the flank or to the proximal two-thirds of a wing bud, scales differentiated as usual in the grafted limbs on the 11th day; 24-48 hours later, feathers began growing from the distal ridges of the scales, especially those of the fourth digit, the first digit, and the metatarsus. Feather growth progressively interfered with, and eventually replaced, some of the scaled areas. Thus it appeared that a new stimulus for the formation of feathers reached the dermis of the graft after the graft dermis had directed the overlying epidermis toward the formation of scales, although the time and nature of the second stimulus were difficult to determine. The stimulus might be
chemical in nature, or it may come from the migration of foreign mesenchyme cells into the locus of the graft. These experiments also support the possibility that leg skin is bipotential.

Thus, from the large number of experiments which have been devoted to the problem of development of epidermal derivatives, several facts emerge: the inductive activity in the formation of limbs comes initially from the mesoderm; in the formation of integumentary derivatives, the inductive factor appears to be located in the dermis. There is evidence in recent experiments that the leg skin may be bipotential, i.e., able to form both scales and feathers with the proper stimulus.
MATERIALS AND METHODS

The technique of chorio-allantoic grafting, as developed by Hoadley (1924) and Willier (1924), was employed in the study of the development of integumentary derivatives in tissues excised from the body of the embryo.

Host eggs, after 10 days of incubation, were candled to determine the location of one of the large chorio-allantoic blood vessels. Using sterile technique, an opening was made with a hack-saw blade in the host shell over the selected vessel. The shell piece was carefully removed without damaging the underlying shell membrane. By making small openings in both the exposed shell membrane and the air-sac end of the shell, and by applying suction to the air-sac with a rubber bulb, the chorio-allantoic membrane was dropped from the overlying shell membrane to which it normally closely adheres. Dropping the chorio-allantoic membrane has the two-fold purpose of allowing the removal of the shell membrane without damaging the chorio-allantoic blood vessels, and leaving more room for the development of the graft inside the host egg.

The grafts were prepared from various combinations of entire skin (dermis and epidermis) plus some type of deeper tissue. Different stages of development and different areas of the body were used in the various grafts, but in all of them, one of the tissues came from a scale-forming area, while the other tissue came from a feather-forming area.

In preparing the donor skin, the area of the embryo which was to be used was excised in sterile chick Ringer's solution. The entire skin was
carefully removed from the underlying tissue using fine steel needles embedded in glass handles, and the deeper tissue was discarded. The donor deep tissue was obtained in a similar manner, except that the skin was discarded and the deep tissue retained.

The two pieces of tissue were combined by wrapping the deep tissue inside the skin, and pressing the two edges of the skin together to form a seal around the enclosed tissue. The combination was carefully transferred to the host chorio-allantoic membrane with a medicine dropper. If the grafts came apart in the transfer, they could usually be recombined on the membrane, using steel needles; once on the membrane, the grafts were held together by surface tension forces. After the removal of excess Ringer's solution with a micropipette, the graft was carefully maneuvered to a position directly over the large chorio-allantoic blood vessel. The openings in the host shell were covered with Scotch tape and the grafts were allowed to develop undisturbed for a period of seven to eight days at 37°C; at the end of this time the graft was recovered and carefully examined in chick Ringer's solution.

In selecting ages of tissue to be used in the grafts, several factors were taken into account. First, in embryos younger than five days the dermis has not differentiated to the degree that it can be separated intact from the deeper tissues. Secondly, skin was chosen at stages in which the inductive specificity of the dermis had not been characterized by previous studies.

The grafts which were made can be divided into three main groups: those in which indifferent "feather skin" was used, those in which
indifferent "scale skin" was used, and those in which skin having scales was used. The grafts using indifferent "feather skin" were designed to test the effect of deep tissue from a scale area on the specificity of dermis from a normally feathered area, from the time at which it is present as a separable layer until just before feather development begins. The skin was taken either from the neck, which normally forms feathers at 6½ to 7 days, or from the wing, which forms feathers at 7½ to 8 days. The deep tissue was obtained by removing skin from the lower leg and cutting out a small piece of prospective muscle, connective tissue, and sometimes prospective bone.

A comparable set of grafts was made using indifferent "scale skin," in order to test the effect of deep tissue from a feather area on scale dermis, from the time shortly after dermal differentiation to the time just previous to the formation of scales. The skin was taken from the lower leg at various ages, and the deep tissue was taken from a variety of sources, most of which normally served as substrate for "feather skin."

The third group of grafts was made using leg skin in the process of scale formation. The reasons for making these grafts were based on the results of the experiments of Amprino and Camosso (1959b); these investigators found that, when the tip of a leg bud was grown on the stump of a wing bud, feathers grew from "scale skin" after the scales had begun to develop. It was thought that changing the substrate of leg skin which was forming scales might evoke a bipotentiality in the skin; such
bipotentiality has been suggested by several authors (Amprino and Camosso, 1959b; Rawles, 1960-61).
RESULTS

The grafts were generally prepared in sets of ten, and, out of each set, one or two grafts were usually unsuccessful. This was either because the host embryo died before the graft was recovered or because the graft was dislodged from the blood vessel and consequently failed to develop. Tissues for grafting were prepared so that the longest dimension was no more than two or three mm; upon recovery, the successful grafts often measured five or six mm in length.

If scales had formed on the graft, they had in most cases developed to the stage of overlapping, and were consequently quite easily distinguished; in cases in which foot skin had been used, claws and foot pads were usually present. The feathers which formed were of variable length (less than 1 mm to several mm) and diameter, and differed in number and distribution on the grafts. Invariably, however, each feather had a noticeable axial artery passing through its entire length, which aided in the analysis of the grafts.

The various combinations using indifferent "feather skin" are summarized in Table 1.

The majority of these grafts, when recovered after seven or eight days, were covered with numerous, normally-developing feather germs. Frequently, the feathers appeared to be inhibited in a small area of the graft; in a few cases, the feathers of the entire graft were blunt and poorly developed. There was no apparent correlation between the normality of feather development and the age of the substrate tissue; that is, inhibition was found in conjunction with both the youngest and the oldest
Table 1. Indifferent feather skin plus deep leg tissue

<table>
<thead>
<tr>
<th>Skin source</th>
<th>Age in days</th>
<th>Deep tissue source</th>
<th>Age in days</th>
<th>No. of cases*</th>
<th>Results</th>
</tr>
</thead>
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<td>Feathers</td>
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<td>Lower leg</td>
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<tr>
<td>Wing</td>
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<td>Lower leg</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Wing</td>
<td>6</td>
<td>Lower leg</td>
<td>8</td>
<td>3</td>
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</tr>
<tr>
<td>Wing</td>
<td>6</td>
<td>Lower leg</td>
<td>9</td>
<td>1</td>
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</tr>
<tr>
<td>Wing</td>
<td>6</td>
<td>Lower leg</td>
<td>10</td>
<td>2</td>
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<tr>
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<td>Lower leg</td>
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<tr>
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</table>

*Refers to grafts recovered from living hosts.

Ages of leg substrate tissue. A correlation was found, however, between adequacy of vascularization and extent of feather development. Grafts which, at the time of recovery, were either to one side of a large vessel or on a very small blood vessel were found to have feathers which were
short and blunt or abnormally slender. In no case was there any sign of
scales on any of the grafts.

The graft combinations made using indifferent "scale skin" are
summarized in Table 2.

The first few sets of grafts using 6- and 7-day lower leg skin gave
variable results; some of the grafts formed only scales, while most

<table>
<thead>
<tr>
<th>Skin source</th>
<th>Age in days</th>
<th>Deep tissue</th>
<th>Age in days</th>
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<th>Results</th>
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<td>Wing</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
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<td>Wing</td>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
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<td>Wing</td>
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<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
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<td>Wing</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
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<td>Wing</td>
<td>8</td>
<td>2</td>
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</tr>
<tr>
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<td>Wing</td>
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<td>3</td>
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</tr>
<tr>
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<td>Wing</td>
<td>10</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
<td>10</td>
<td>Liver</td>
<td>10</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
<td>10</td>
<td>Brain</td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
<td>10</td>
<td>Neck</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td></td>
<td></td>
<td>106</td>
<td>78</td>
</tr>
</tbody>
</table>

*Refers to grafts recovered from living hosts.
formed both scales and feathers. Because of the difficulty at such an early stage in distinguishing the boundary between the upper leg, which will form feathers, and the lower leg, which will form scales, the possibility of artefact was considered. To test for this, control grafts were made, in which the lower part of the leg was removed and grown intact on the chorio-allantoic membrane for a week or eight days. The presence of feathers on the upper part of the control grafts indicated that the skin used in the experimental grafts had been taken from not only the scale-forming areas, but from a small part of the feather-forming area as well. Subsequent grafts were made using only skin from the tip of the leg bud. These grafts invariably formed scales, claws and foot pads, although in many cases scale development appeared to be inhibited. It should be noted that trying to remove the skin from the tip of the leg bud at this stage is extremely difficult, so that in most cases parts of the deep leg tissue remained attached to the isolated skin.

The grafts using 9- and 10-day leg skin formed well-developed, overlapping scales in all cases, with no sign of feather development and very little inhibition of scale development.

Table 3 summarizes the grafts made with leg skin which was already forming scales.

The majority of the grafts in this series were covered with well-developed, overlapping scales after the usual incubation period. The scales were generally large and flat on one side of the graft and small and rounded on the other side, in accordance with normal local variations.
Table 3. Skin having scales plus various deep tissues

<table>
<thead>
<tr>
<th>Skin source</th>
<th>Age in days</th>
<th>Deep tissue source</th>
<th>Age in days</th>
<th>No. of cases*</th>
<th>Feathers</th>
<th>Scales</th>
<th>Both</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower leg</td>
<td>11</td>
<td>Wing</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
<td>12</td>
<td>Wing</td>
<td>5</td>
<td>8</td>
<td>0</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Lower leg</td>
<td>12</td>
<td>Wing</td>
<td>6</td>
<td>8</td>
<td>0</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Lower leg</td>
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<td>Wing</td>
<td>7</td>
<td>23</td>
<td>0</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Lower leg</td>
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<td>Wing</td>
<td>8</td>
<td>11</td>
<td>0</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Lower leg</td>
<td>12</td>
<td>Wing</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
<td>12</td>
<td>Neck</td>
<td>7</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
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<td>Brain</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
<td>12</td>
<td>Liver</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Lower leg</td>
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<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Fourth toe</td>
<td>12</td>
<td>Wing</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Fourth toe</td>
<td>12</td>
<td>Wing</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Fourth toe</td>
<td>12</td>
<td>Wing</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Fourth toe</td>
<td>12</td>
<td>Wing</td>
<td>8</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td></td>
<td></td>
<td>89</td>
<td>77</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

*Refers to grafts recovered from living hosts.

In scale morphology.

In a few cases, however, both feathers and scales formed from the scale skin; the combinations which sometimes resulted in this type of development all involved 12-day leg skin and wing tissue of various ages. In these cases the feathers were generally few in number and fairly small. They were found in close association with the scales, and in some cases appeared to be growing from the scales. Where more than one feather formed, they seemed to be located in the same general area of the graft.

As controls, grafts were made from isolated 12-day leg skin, from 7-day deep tissue of the neck, and from 7-day deep tissue of the wing.
The isolated leg skin formed only scales in all cases, while the deep tissues formed a smooth outer layer devoid of derivatives of any sort. These data suggest that the results of this set of grafts are valid.
DISCUSSION

Before discussing the results of the grafting experiments, it might be well to review the purpose and background of the experiments. In a number of investigations, Saunders found that 3- to 4½-day mesoderm was able to determine the regional character of epidermal development; at this stage a differentiated dermal layer is not present. Sengel (1958) showed that the developmental pathway of skin which was at the stage of dermal condensations was specified by the dermis; in order for the dermal condensations to form, however, living substrate tissue was necessary. Between the time at which the dermis is differentiated (4½ to 5 days) and the time at which outgrowth begins (6½ to 7½ days for feathers; 10 to 11 days for scales) the source of specificity has not been determined. The grafting experiments described in this dissertation represent an attempt to determine the source and time of origin of dermal specificity.

The grafts in which 5- to 6-day skin from a normally-feathered area was combined with deep tissue from the lower leg resulted in the formation of feathers in all cases. The age of the leg tissue ranged from five days, shortly after dermal differentiation, to 12 days, after scale formation had been initiated. If the deep tissue of the leg has any effect on specificity, some type of influence should have been apparent with at least one of the stages used. The "feather skin" did not seem to be affected in any manner by the presence of "foreign" substrate tissue, since it developed normally. Such results imply not only that the deep tissue of the leg exerts little or no influence on "feather skin"
but that the dermis of the "feather skin" can specify the formation of feathers in the epidermis as early as 5 days, regardless of the substrate tissue present. If this is true, then the dermis must be capable of such specification previous to the age at which it is capable of exerting its inductive effects on the epidermis (Sengel 1956). The results also suggest that deep leg tissue is a suitable evocator of dermal inductivity in "feather skin;" this concept is in accordance with the non-specific substrate role proposed by Sengel (1957b).

Graft combinations utilizing 6- to 10-day leg skin wrapped around a variety of deep tissues gave comparable results. Except in those cases mentioned in the results, leg skin of these ages formed only scales. Most of the deep tissue was taken from the neck and wing of embryos at various ages; such tissue would ordinarily be found underlying feathered skin. Brain and liver tissues of several different stages were used in a few of the grafts, because of the possible effects of their rapid metabolic activities on the development of the skin. Since deep wing and neck tissue, as well as brain and liver, had no effect on development of scales in leg skin, it is probable that these tissues play little or no role in the specificity of the dermis. In addition, the fact that the leg dermis is apparently unresponsive to the deep tissues suggests that it is innately capable of specifying scale formation. As in the last experiments, the results of these grafts imply that the various deep tissues used provide a suitable substrate for evocation of dermal inductive powers.

Variable results were obtained with the series of grafts using 12-
day leg skin, which is already in the process of forming scales, and deep tissue from a variety of ages and sources. In most cases normal scales developed in the grafts, indicating that the pattern of scale development was unaffected by the deep tissue. In a few cases in which 5- to 8-day deep tissue of the wing was used, a few feathers developed in the grafts, suggesting that a developmental bipotentiality might exist in leg skin, as proposed by Amprino and Camosso (1959b) and Rawles (1960-61). Because so little is known about such a possibility, and because the grafting experiment contributed only a small amount of evidence in favor of such a mechanism, a few speculations are all that can be made. It is difficult to explain the fact that, when 12-day leg skin was used in the grafts, feathers formed in a few cases, while, when younger leg skin was used, no feathers formed. A possible explanation is that leg skin is receptive to a feather-forming stimulus from deep tissue only after scales have been induced to form in the epidermis, and that the deep tissue exerts its effects on the bipotential dermis, only when recently removed from the normally feathered area. In other words fresh deep tissue from a normally feathered area and skin in the process of forming scales may both be necessary components for the evocation of bipotentiality. It is possible that a combination using ages other than those used in these grafts (i.e., older leg skin and/or younger deep tissue) would yield a greater percentage of grafts showing both types of derivatives. The possibility that leg skin can be affected by a feather influence only after scales begin developing is supported by the work of Amprino and Camosso (1959b), who found that feathers grew from scales one or two days after
scales became evident in the leg skin. The possibility that the leg dermis is a weak scale inductor (Rawles 1960-61) may also play a role in the phenomenon of bipotentiality, since the potential for scale formation might thus be more easily affected by feather influences.

Based in part on these experiments and in part on the work of other investigators, the following hypothesis is proposed: (1) specificity of the dermal layer is established at the time of its inception, (2) deeper tissues are a necessary non-specific evoker of the dermal inductive specificity, and (3) scale dermis might be bipotential, being able to form both scales and feathers under certain circumstances. Each of these points is discussed below.

The results of the grafting experiments using indifferent "feather skin" suggest that the dermis is innately specific, since deep tissue from scale areas has no apparent effect on the inductive specificity of newly differentiated feather dermis, even when very young tissues are used. Likewise, feathers cannot be induced to form in indifferent leg skin by placing it on a substrate of deep tissue from feather areas. Apparently the specificity which is shown by the mesoderm of a 3- to 4½-day limb bud (Saunders 1958) is shown mainly by the dermis, as soon as this layer has differentiated. In regard to skin which is in the process of forming derivatives, conclusive evidence has been presented (Sengel 1958; Rawles 1960-61) that the dermis is responsible for specificity.

Evocation of the dermal inductive powers by deep tissue is suggested primarily by the work of Sengel (1957b), who found that skin younger than
the stage of mesodermal condensations was unable to form integumentary derivatives when isolated and grown in tissue culture. If, however, the skin was placed on a substrate of tissue from any one of a number of organs, feathers formed in the indifferent skin. Thus the deep tissue apparently acts just before the time of outgrowth, thereby enabling the dermis to form condensations and to induce the epidermis to form derivatives. The fact that such an effect is relatively non-specific is demonstrated by the wide variety of tissues which will produce the same result. In the grafting experiments it was found that a variety of ages and sources of substrate tissue were apparently suitable for the evocation of integumentary derivatives in indifferent skin.

Because leg skin, under certain conditions, is able to form both scales and feathers, several authors have suggested that leg dermis is bipotential. The factors which, in the various investigations, have elicited the expression of bipotentiality are quite diverse: replacing normal leg epidermis with epidermis which contains young feather germs (Rawles 1960-61), growing the leg bud tip on the stump of the wing bud (Amprino and Camosso 1959b), and, in this investigation, changing the dermal substrate. The fact that, in the experiments of these investigators, the pattern of feather growth eventually dominated the scale pattern suggests that feather induction is a stronger force than scale induction. If such a phenomenon as bipotentiality is indeed responsible for these results, much more work is needed before the mechanism involved can be predicted with any assurance.

In order to test this possibility further it would be useful to
continue the investigation with experiments which vary not only the inter­
action between dermis and epidermis, or between deep tissue and dermis,
but which separate all three components and grow them in various
combinations of ages and sources. If scales can be made to grow in tissue
culture, such a technique would be best suited for such studies.

The suggestions which have been presented can be summarized in the
following diagram:

Diagram 2. Hypothesized mechanism of interaction
PART II: HISTOCHEMISTRY
REVIEW OF LITERATURE

Ribonucleic Acid

For many years before the elucidation of the role of RNA in protein synthesis, investigators noticed the presence of this substance in differentiating tissues. Brachet (1947a) found RNA widely distributed in the tissues of the chick embryo. Novikoff and Potter (1948) measured nucleic acid concentrations during the development of the chick and found that RNA reached a maximum during the period of maximal growth. The accumulation of RNA during epidermal keratinization was noted by Caspersson (1950). In the larval salivary glands of Drosophila, Lesher (1951) found that RNA was abundant in the epithelial cytoplasm only when the cells were actively secreting and decreased in post-secretory stages. Brody (1952) noticed that in the development of the human placenta, RNA accumulated just before the period of most rapid growth.

In the last few years it has been shown conclusively that RNA plays a direct role in the synthesis of protein. There appear to be three different types of RNA operating in the process: messenger RNA, an unstable form which carries coded information from nuclear DNA to the cytoplasm (Brenner, Jacob, and Meselson 1961; Hurwitz and Furth 1962); ribosomal RNA, long-chain molecules along which the forming protein molecules are assembled; and transfer RNA, which carries activated amino acid residues to the template RNA (Hoagland et al. 1957). Several excellent reviews of the mechanisms involved (Simkin 1959, Hoagland 1959, Allfrey and Mirsky 1961) establish the nature of the relationship between ribonucleic acid
and protein synthesis.

The possible relationship of RNA to embryonic induction was suggested by Brachet (1947b), who found that ribonucleoprotein, especially RNA, was necessary in evokers for inductive activity. McKeehan (1956) observed that, during lens induction in the chick, the highest concentration of RNA seemed to pass from the retina to the cells of the overlying ectoderm. During neural induction in the frog embryo, Rounds and Flickinger (1958) found that mesodermal RNA decreased while the RNA level of the ectoderm remained constant. Hayashi (1959) studied guinea pig liver ribonucleoprotein and concluded that the protein component of the material seemed to be the active factor for regional induction, since ribonuclease destroyed the RNA but not the inducing power. Kawakami et al. (1960a) found that a fraction of chick embryo extract, rich in ribonucleoprotein, was able to induce spinocaudal structures in ectoderm of the Triturus gastrula. When the proteins were separated on a column, varying degrees of mesodermal and neural induction were obtained with each of the eight fractions. These results suggested that the protein portion of the ribonucleoprotein was the active inductive agent (Kawakami et al. 1960b). Lash et al. (1957) found that chick embryonic spinal cord and notochord were able to induce the formation of cartilage in chick somite cells, through a millipore filter. Attempts to isolate the active inductive factor led to the extraction from the notochord and spinal chord of a nucleotide-containing fraction which could induce cartilage formation in explanted somites (Lash et al. 1962). These results suggest that the RNA of the ribonucleoprotein was the active
agent in induction.

Protein

The formation of the proteins of the integument has not been extensively studied. Rogers (1959) suggested that transition from the stage of keratin formation to the cornified stage involves not only the keratin molecules, but a second protein or "cement" substance. In his study of chick epidermal differentiation in a chemically-defined medium, Wessells (1961a) found that the process consisted of three consecutive events: formation of primary and secondary periderm containing kerato-hyaline granules (5-10 days); the appearance of birefringent, fibrous, sulfur-rich structures in the subperiderm (11-15 days); and cornification, during which the fibrous material combined with a cementing substance (16-17 days).

Polysaccharides

There appear to be at least two different types of mucopolysaccharide commonly found in embryonic tissue, acid or metachromatic mucopolysaccharide and neutral or PAS-positive mucopolysaccharide (Montagna et al. 1952a; Moog and Wenger 1952), although Lillie (1950) believed these two substances to be the same.

Glycogen, a neutral polysaccharide, has been found in regenerating epidermis (Bradfield, 1951) and in the growing hair follicle (Montagna et al. 1952b). Montagna suggested that glycogen was deposited in areas where damming of keratinized material had retarded the normal rate of
keratinization. Bradfield (1951) and Wislocki and Dempsey (1945) believed that glycogen was found in active regions having only a limited blood supply, where anaerobic oxidations were necessary for maintenance of cells.

Acid mucopolysaccharides have been found in many mammalian tissues (Bunting 1950), where they are believed to serve a nutritive function as well as being protective in certain areas. Michaelis (1944) believed that acid mucopolysaccharides were half-esters of sulfuric acid combined with high molecular weight carbohydrates, such as chondroitin sulfate. Sylvén (1950) found metachromasia in the papillae of actively growing hair follicles and basal regions of the epidermis and suggested that the acid mucopolysaccharides were labile sulfur-containing groups involved in the synthesis of keratin.

In recent years acid mucopolysaccharides have been shown to play an important role in the differentiation of chick embryonic cartilage (Lash et al. 1960), morphogenesis of the palate of the mouse embryo (Walker 1961), and development of feather germs in the chick embryo (Sengel et al. 1962). These three groups of workers studied the acid mucopolysaccharides through staining and through autoradiography, in which the incorporation of labeled sulfate was followed.

Mitochondria

Although a great deal of work has been done recently on the fine structure and biochemistry of adult mitochondria, very little work has been done on the mitochondria of vertebrate embryos. Carey and Greville
(1959a) worked out a procedure for the isolation of mitochondria of chick embryos. They found (1959b) that mitochondria from 5-day chick embryos contained enzymes required by the tricarboxylic acid cycle, but not those necessary for fatty acid oxidations. North and Pollak (1961), using the electron microscope, found that the embryonic cells of the chick liver had a greater number of mitochondria in association with the nucleus than the adult cells, suggesting a correlation between the associations of cell organelles and the control of cellular metabolism. Hamilton and Kischer (1961) found that the mitochondria in the epidermis of differentiating feathers were longer than those in the other epidermal zones. They suggested, because of the localization of basophilia in the mitochondria, that RNA was associated with mitochondrial activity during morphogenesis.

Alkaline Phosphatase

A plethora of literature on the location and function of alkaline phosphatase (AP) has been written, yet even today this enzyme is not well-understood. Most investigations have involved staining with the Gomori method (Gomori 1946), a technique which could lead to misinterpretation due to diffusion of the reaction (Gomori 1950). The likelihood of diffusion artefacts was discounted by Danielli (1946) on the basis of comparison with other techniques. Chèvremont and Firket (1953) found that diffusion of phosphatase and adsorption by nuclei could occur near cytoplasmic structures especially rich in the enzyme and suggested that the possibility of artefact could be ruled out only when extreme care is
given to technique. Thus, the literature must be interpreted with this in mind.

The importance of AP in various developmental processes has been extensively studied. The enzyme has been found throughout development of the chick embryo (Moog 1944), during organogenesis in amphibian embryos (Brachet 1946), and during early development of the rat and mouse (Mulnard 1955). The role of phosphatase in the development of the down feather was investigated by Hamilton and Koning (1956) and Hinsch (1960). Hamilton and Kischer (1961) found that, if AP were added to tissue cultures of chick back skin, the feathers which developed grew more rapidly and attained greater length. The enzyme has also been found in developing chick muscle (Konigsberg and Herrmann 1955), in the ectodermal tips of developing rat limb buds (McAlpine 1956), in the developing thyroid gland (Dempsey and Singer 1946), and in the developing thyroid, parathyroid, and thymus glands of the rat (McAlpine 1955). AP has also been correlated with luteal growth in the rat (Malone 1960), with development of the intestinal epithelium during amphibian metamorphosis (Chieffi and Carfagna 1960), and with regeneration of the feathers of canaries (Kobayashi and Maruyama 1957). Thus AP appears to play a definite role in differentiation and growth.

Numerous hypotheses have been proposed to explain the function of AP. Fell and Danielli (1943), as a result of their study of healing wounds, suggested that AP was needed in the formation of structural proteins such as collagen. The presence of phosphatase on forming collagen fibers was believed by Gold and Gould (1951) to be due to
adsorption and retention as a part of the healing process. Robertson et al. (1950) were unable to find AP on collagen fibers during formation.

Hardy (1952) found AP in the dermal papilla of the hair follicle and suggested a role in the transport of organic substances between papilla and hair matrix during differentiation and growth. Danielli (1953) suggested that alkaline phosphatase acted in transport as a phosphokinase, releasing energy by breaking high energy phosphate bonds. The energy would then be available for contraction of proteins in the cell membrane, bringing a variety of organic substances into the cell.

The presence of AP in the developing tooth led Bevelander and Johnson (1945) to suggest that it played a role in calcification. Fleisch and Neuman (1960) believed that the enzyme was important in calcification because of its ability to remove inhibitory substances from the serum.

In a study of nuclear alkaline phosphatase, which increases during early development, it was suggested that the enzyme might act in synthesis of DNA and thereby play an active and essential role in mitosis (Chêvremont and Firket 1953).

Whether AP is a single enzyme or a group of related enzymes has been questioned by several workers. Newman et al. (1950) found that several enzyme groups were able to liberate phosphate at pH 9.2. Moog (1959) proposed that several related phosphatases might be present, in different locations and at different times. In the differentiation of the duodenum of the fetal guinea pig, Moog and Ortiz (1960) found that increase of phosphatase was accompanied by an increasing preference for phenyl
phosphate, rather than beta-glycerophosphate, as a substrate, suggesting a change in the type of phosphatase being synthesized. Hinsch (1960) found that at least two phosphatases in the developing down feather were capable of using various substrates at pH 9.0-9.4. Moss et al. (1961) isolated AP from human bone, liver, kidney, and intestine and found that there were small differences in the Michaelis constants of the phosphatases of individual tissues, suggesting that each tissue might have a characteristic AP. Electrophoresis on starch-gel resolved the enzyme from each individual tissue into one major and several minor components or isozymes (Moss and King 1962). Unlike the isozymes of lactic dehydrogenase which are distinct proteins (Markert and Ursprung 1962) and are believed to play a role in differentiation, the alkaline phosphatase isozymes differed only in mobility.

**Relationship between Alkaline Phosphatase and Ribonucleic Acid**

Alkaline phosphatase and ribonucleic acid have been found in association in a variety of tissues. Rhodes and Dalton (1961) found that cells of differentiating urodele pituitaries were rich in phosphatase and RNA only after the gland became functional, at which time both substances appeared at approximately the same time. Close association in time and location were found for AP and RNA in the developing down feather of the chick (Koning and Hamilton 1954) and in the developing juvenile wing feather of the chick (Koning 1957). Rogers et al. (1960) found that AP in the chick brain increased sharply at the onset of rapid synthesis of RNA and protein. Johnson and Bevelander (1954) demonstrated the
coexistence of AP and RNA in both time and location in the odontoblasts and ameloblasts of the developing tooth. On the other hand, an inverse relationship of the two substances was found in the enamel organ of the developing rat tooth (Symons 1956) and in the rodent placenta (Wislocki et al. 1946).

The significance of such a relationship during development has been discussed by several investigators. AP might be part of an enzyme system involved in the liberation of newly synthesized protein from a complex with nucleic acid (Bradfield 1950; Lesher 1952). The enzyme is also able to liberate organic phosphate from RNA, depolymerized DNA, and mononucleotides (Schmidt et al. 1956; Schmidt 1959). According to Krugelis (1946) AP is able both to split and synthesize nucleic acids, although Moog (1946) suggested that the enzyme was hydrolytic only, rather than both hydrolytic and synthetic. Hamilton and Koning (1956) believed that AP expedited RNA synthesis by splitting esters and transporting certain substances across the basement membrane for construction of RNA. Gibley (1959) suggested that AP provided energy to be used by RNA for synthesis of protein.

Relationship between Alkaline Phosphatase and Glycogen

Because of the relationship found in tissues between phosphatase and glycogen, and because phosphorylated intermediates are known to exist in carbohydrate metabolism, the functional relationship of these two substances has been questioned. Several investigators have suggested that AP play a role in phosphorylation and dephosphorylation during
glycogenesis and deposition of glycogen in tissues (Wislocki and Dempsey 1945; Johnson and Bevelander 1946, 1947). Moog (1946) and Harris (1932) suggested that the enzyme liberates phosphate groups through glycolysis, the phosphate radicals then being used in the formation of bone. Lesher (1952) and Moog and Wenger (1952) believed that the neutral mucopolysaccharide acted as an orienting cytoskeleton for the phosphatase, as well as providing a suitable environment for its activity.
MATERIALS AND METHODS

Legs to be used for histochemical studies were removed from living White Leghorn chick embryos by cutting through the tibia-fibula just above its junction with the tarso-metatarsus, thereby including all of the scale-forming and a small part of the feather-forming areas of the leg. In a few cases the foot was removed, but in general the amputated leg was left intact. The legs were washed briefly in chick Ringer's solution, then placed immediately in the fixative. For each of the techniques used, legs were fixed at stage 36 (10 days), stage 37 (11 days), stage 38 (12 days), stage 39 (13 days), and stage 40 (14 days) (Hamburger and Hamilton 1951), thereby including most of the period during which scales are being formed.

After fixation, all of the tissues were dehydrated in a graded series of alcohols, cleared in benzene, and embedded in a mixture of ten parts paraffin (m.p. 56-58° C) and one part bayberry wax. Sections were cut with a rotary microtome, at five microns for the demonstration of mitochondria and at seven microns for all other techniques. An albumin fixative was used to attach sections to slides in preparation for staining. In procedures requiring the use of control sections, the test and control slides were made from adjoining pieces of the same ribbon. Each procedure was repeated on sections from at least five different legs at the same stage, in order to be sure that results were reproducible.

Details of the fixation and staining procedures varied according to the technique and are discussed individually below.
Ribonucleic Acid

Tissues to be stained for ribonucleic acid (RNA) were fixed overnight in a mixture of 95 parts absolute alcohol and 5 parts glacial acetic acid (Wolman and Behar 1952). After embedding, sectioning, and mounting, the slides were deparaffinized, hydrated, and stained for fifteen minutes in a 0.05 percent solution of toluidine blue in 5 percent alcohol. The sections were destained overnight in tertiary butyl alcohol (Montagna et al. 1952a), cleared in xylol, and mounted in Hartman-Leddon Synthetic Resin (HSR). In order to demonstrate the presence of ribonucleic acid, half of the slides were incubated at 58° C in a 0.1 percent solution of ribonuclease in distilled water for three hours (Brachet 1953); the other slides were incubated for the same period of time at 58° C in distilled water. Areas of tissue which failed to stain after enzymatic treatment were presumed to have contained RNA.

Acid Mucopolysaccharides

Metachromatic staining by the toluidine blue technique discussed above demonstrates the presence of acid mucopolysaccharides, since the pink color tends to accumulate around the specific irregularities of the mucopolysaccharide molecule (Michaelis and Granick 1945).

Alkaline Phosphatase

Alkaline phosphatase was demonstrated in the sections using the technique of Gomori (1946). Tissues were fixed overnight in cold acetone.
and were cleared and embedded as rapidly as possible, in order to minimize destruction of the enzyme. The sections of tissue were deparaffinized, hydrated, and incubated for $1\frac{1}{2}$ hours at $37^\circ$ C in a substrate mixture containing 25 ml distilled water, 10 ml 2 percent sodium barbital, 10 ml 2 percent sodium glycerophosphate, 5 ml 2 percent calcium chloride and 1 ml 2 percent magnesium sulfate. Control slides were incubated for $1\frac{1}{2}$ hours at $37^\circ$ C in a similar mixture which lacked only the sodium glycerophosphate. The slides were washed in distilled water, immersed in 2 percent cobalt chloride for 5 minutes, washed again in distilled water, and treated with ammonium sulfide for 5 minutes. The development of a black precipitate of cobalt sulfide in the glycerophosphate-treated slides indicated sites of enzymatic activity. The slides were washed, dehydrated, cleared in xylol, and mounted in HSR.

Mitochondria

Tissues to be stained for mitochondria using Regaud's technique (Davenport 1960) were fixed for four days in a solution containing 5 parts of 40 percent formaldehyde, 8 parts of 7.5 percent potassium dichromate, and 12 parts of distilled water; tissues were transferred to fresh fixative on each of the four days. Following fixation, tissues were washed in 3 percent potassium dichromate for a period of $3\frac{1}{2}$ days, the solution being changed six times during this time interval. After washing overnight in running tap water, the tissues were dehydrated, cleared, embedded, and sectioned at 5 microns. After deceration and hydration, the slides were immersed in a 4 percent iron alum mordant for
24 hours, rinsed in distilled water, and stained for 24 hours in a 2.5 percent chemically ripened solution of iron haematoxylin in 5 percent alcohol. The sections were destained in 2 percent iron alum under the microscope, until the proper degree of differentiation was obtained. The sections were then washed, dehydrated, cleared, and mounted in HSR.

Neutral Polysaccharides

Neutral polysaccharides were localized in the sections using the periodic acid-Schiff (PAS) method (McManus 1948; Hotchkiss 1948), in which oxidation by periodic acid leaves reactive aldehyde groups available for staining with Schiff's reagent. Tissues to be treated by the PAS technique were fixed overnight in acid-alcohol, and embedded and sectioned in the usual manner. After decration and hydration, the slides were immersed in 0.5 percent periodic acid for 10 minutes, washed in running tap water, and stained in Schiff's reagent for 15 minutes. The slides were then passed through three two-minute changes of 0.5 percent sodium bisulfite, and were washed, dehydrated, cleared, and mounted.

In order to demonstrate the presence of glycogen, some of the slides were placed in a 0.001 percent solution of diastase (Davenport 1960), containing a trace of sodium chloride, at 37° C for one hour; control slides were incubated in distilled water for the same period of time at 37° C. Areas which were stained pink in untreated sections but not in diastase-treated ones were considered to contain glycogen.
Proteins

Tissues in which proteins were to be stained were fixed overnight in acid-alcohol. After embedding and sectioning of tissues, the slides were deparaffinized in xylol, passed through absolute alcohol to 95 percent alcohol, and stained for fifteen minutes in a solution containing 10 g of mercuric chloride, 100 mg of bromphenol blue, and 100 ml of 95 percent alcohol. The sections were then washed in 0.5 percent acetic acid for 20 minutes, washed in water for two minutes to convert the dye to its blue alkaline form, rapidly dehydrated, cleared and mounted (Mazia, Brewer, and Alfert 1953).

Keratin

An attempt was made to demonstrate the localization of keratin, by the staining of protein-bound sulfur in the sections. Tissues were fixed in acid-alcohol; after embedding and sectioning, slides were deparaffinized, dehydrated, and passed through a 1 percent solution of collodion. Oxidation of the disulfide linkages of cystine was carried out using a 20-minute treatment with 0.5 percent potassium permanganate (Lillie et al. 1954). The brown crystals of permanganate were removed by rinsing in 1 percent oxalic acid, followed by a ten-minute rinse in running tap water. The sections were stained for one hour in 0.05 percent methylene blue in 0.1N hydrochloric acid, dehydrated in acetone directly after staining, cleared, and mounted.
RESULTS

Before a description of the results of the various histochemical procedures is given, a few words should be said concerning regional differences in time of scale formation. In this study it was found that scale development was initiated first on the anterior side of the leg (stage 36½ to 37), then on the lateral areas (stage 37½ to 38), and finally on the posterior part of the leg (stage 38½ to 39). Scales developed more rapidly on the lower anterior part of the leg than on the upper anterior part. In order to maintain a relative degree of constancy, the results are given for the most rapidly developing scales, those on the lower half of the anterior side of the leg.

General Morphology

At stage 36 (10 days) there is no evidence of scale formation, and the entire epidermis consists of a single row of basal cells and a single row of flattened peridermal cells. The scale arises from a thickening of the epidermis, brought about by an increase in the size of the basal layer and a doubling of the periderm layer (Figure 1). This double layer probably corresponds to the primary and secondary periderm layers described by Wessells (1961a).

By stage 37 (11 days) there are definite protrusions localized in prospective scale areas. The epidermis of the protrusions is obviously thickened, having approximately two rows of cells in the epidermis proper and a double row of flattened cells in the periderm; in non-scale areas, no epidermal thickening is apparent. The dermis at this stage is also
thickened, having a greater cell density in prospective scale regions (Figures 3 and 16).

The developing scales are beginning to bend in a downward direction by stage 38 (12 days). In the scale areas, the epidermis consists of at least two layers of cuboidal cells and two layers of flattened periderm cells. The concentration of dermal cells has moved toward the direction of bending, and is localized in the future apex of the scale (Figures 7 and 17).

By stage 39 (13 days) the developing scales are beginning to overlap. The entire epidermal layer is somewhat thickened and consists of at least two peridermal layers and two or three inner layers. The epidermis of the flat surfaces of the overlapping scales has a layer of slightly elongated basal cells, several layers of cuboidal cells, and a double periderm layer. At the apex of the scale, the epidermis is thinner, having approximately one less layer of cuboidal cells. The thickest area is found in the inturned portion of the epidermis, where the basal cells are decidedly columnar. The greatest density of dermal cells seems to be just under the epidermis of the scale apex (Figures 5, 18, and 19).

The epidermis of the overlapping scales of stage 40 (14 days) is thickest at the flat surface of the scale, having thinned out both at the apex and at the inturned area; there also appears to be a denser cell population in the epidermis of the flat surface than in other areas. The periderm appears to have begun keratinization, judging by the smooth outer border and the parallel alignment of the elongated nuclei. The concentration of dermal cells is less dense, and is localized at the base
of the infolding, rather than in the apex (Figure 20).

Ribonucleic Acid

With toluidine blue, all basophilic substances in the tissue are stained a deep blue. If control sections are treated previously with ribonuclease, areas which fail to stain with the dye can be presumed to have been rich in RNA.

In skin from legs having only epidermal thickenings in scale primordia, the RNA is concentrated primarily in the epidermis, just above the basement membrane. The distribution is fairly even along the length of the epidermis, although the concentrations are slightly greater in scale areas (Figures 1 and 2).

By the time that prospective scale areas are marked out by swellings, RNA is definitely more concentrated in these areas. The greatest concentration is just above the basement membrane, although there is also RNA in the area just above the nuclei of the basal epidermal cells. In spite of deep blue staining of the mesodermal condensations, very little of the color is prevented by pre-treatment with ribonuclease; it is probable that the color is due to the large number of nuclei in this area (Figures 3 and 4).

After the scales begin to overlap, the preferential concentration of RNA in the scale areas is more apparent. The RNA is most concentrated above the basement membrane of the area which will form the flat surface of the scale. There is a fairly large amount at the apex and least at the infolded region. Some RNA appears to be localized in the periderm,
at least in the later stages, especially on the flat surface of the scale (Figures 7, 8, and 9).

In stages where overlapping has occurred, the RNA at the basement membrane is very concentrated only on the flat surface, apparently having decreased in the apex and infolded area of the scale (Figures 5 and 6).

Alkaline Phosphatase

Alkaline phosphatase activity, as demonstrated by the Gomorí technique, causes a dark black staining reaction. In skin with only slight thickenings in prospective scale regions, phosphatase is evenly distributed along the dermis in a dark band which is located beneath the basement membrane. Activity is indicated in the nuclei and cytoplasm of the dermal cells and in intercellular areas, but no reaction is shown by the epidermis.

After scale protrusions are formed, alkaline phosphatase is heavily concentrated in the dermal condensations. The centers of enzymatic activity, however, are slightly displaced from the centers of the primordia toward the direction of the future apex; in this area, the concentration is greatest just beneath the basement membrane. In the rest of the scale, activity decreases in gradient form to a minimum in the area of the future base of the scale (Figures 10 and 11).

At the time that infolding is beginning, the enzyme is concentrated throughout the dermis of the scale, especially at the apex. Along the flat portion of the scale a very dark narrow line of heavy activity is present just under the basement membrane (Figures 12, 13, and 14).
In the fully formed scale a similar distribution in the apex and at the basement membrane is apparent. It was also noted in the later stages that the nuclei of the basal epidermal layer give a positive reaction for alkaline phosphatase (Figure 15); this could be due to diffusion from the strong dermal reaction (Chèvremont and Firket, 1953).

Protein

In using a general stain for proteins, it was expected that most areas of the tissue would be stained to some extent; certain regions, however, were found to contain a much higher protein concentration than others.

In general it was found that the protein content of the epidermis is far greater than that of the dermis. In skin showing only swellings where scales will be formed, the protein is preferentially concentrated in these areas. Greatest amounts are found at the basement membrane, where staining is blue-green, and in the periderm, where staining is bright blue (Figure 16).

When overlapping is just beginning, some degree of localization of strongest protein concentration occurs. At the basement membrane the protein is most abundant in the flat portion of the scale and least in the inturned area; although the entire periderm stains darkly, the concentration is greatest at the apex and in the area which is turning under (Figures 17, 18, and 19). In the inturned area of overlapping scales, the periderm is wrinkled, as though it were being compressed (Figures 18 and 19).
In the oldest scales studied very strong reactions for protein were found in the periderm and at the basement membrane of the flat portion of the scale (Figure 20).

Specific staining for keratin revealed epidermal localizations similar to those found with the general protein stain. Throughout development the deepest color is found at the basement membrane, especially in scale areas, and in the periderm. In later stages the greatest concentration of keratin is in the periderm on the flat surface of the scales.

Because this technique utilizes a general stain for disulfide and sulfhydryl groups, its specificity for keratin is questionable. It is probable, however, that the keratin, as well as the keratin precursors, would be stained preferentially because of their high content of these sulfur-containing groups.

Mitochondria

In the stages before scales begin to form, mitochondria are found in the basal layer of the epidermis, primarily below and above, but also lateral to, the nuclei. After the epidermis becomes thickened, a marked increase in the number of mitochondria can be seen in prospective scale areas. These mitochondria are long and thread-like and are in heaviest concentration just above the basement membrane. Because the second greatest concentration is found above the nuclei of the basal cells, the general distribution in the scale areas gives the impression of a double row of mitochondria (Figures 21 and 22).
As development proceeds, the mitochondria become concentrated at the basement membrane of the scale apex and of the flat portion of the scale. In later stages the mitochondria assume the shape of considerably shortened rods (Figure 23).

Acid Mucopolysaccharides

Acid mucopolysaccharides are best demonstrated by the metachromatic reaction of toluidine blue. Throughout the development of scales, this pink color is distributed on a fibrillar network in the dermis, and is especially concentrated at the basement membrane. The reaction appears to be only slightly stronger in scale primordia than in non-scale areas.

Glycogen

Neutral mucopolysaccharides in general are found in a distribution similar to that of the acid mucopolysaccharides, as well as in some additional areas. There is a fibrillar network in the dermis and at the basement membrane, which is PAS-positive and which may be the same as that stained metachromatically.

Neutral mucopolysaccharides are also found in the epidermis, and especially in the periderm, in fairly large concentrations. The prominent PAS-positive globular material which develops in the periderm is removed with diastase and is therefore glycogen.

In skin without scales the glycogen is evenly distributed in the periderm (Figures 24 and 25), but as the scale bulges form, glycogen becomes more concentrated in these areas. When overlapping begins, the
amount of the globular material increases markedly, becoming preferentially located in the periderm of the inturned area of the scale (Figures 26 and 27). In the fully-formed scale, the globules are quite prominent and are found throughout the periderm, although they are still concentrated primarily in the inturned area (Figures 28, 29, 30, and 31).
DISCUSSION

In studying the results of the procedures employed in this investigation, two general observations can be made: (1) most of the various biological chemicals studied are unquestionably related to the process of scale morphogenesis, and (2) in most cases, dermal and epidermal reactions appear to be intimately associated with each other, emphasizing the importance of interactions between these two germ layers.

The relationship between dermis and epidermis is illustrated not only by the histochemical tests, but also by the morphological pattern of scale development. In the earliest stages of differentiation epidermal thickening is accompanied by condensation of dermal cells in the prospective scale area. Bending over of the scales occurs with movement of the dermal condensation toward the future scale apex. In earliest stages of overlapping, the epidermis is thinnest at the scale apex and the greatest number of dermal cells is in the apex; this suggests that rapid mitotic growth of the apical dermis might cause thinning of the epidermis simply by mechanical stretching. In the inturned area of the scale the epidermal layer is thickest, possibly because of compression effects of the infolding. When the scale is fully formed and has begun keratinization, the epidermis is approximately of the same thickness in both the apex and fold; the dermal condensation has moved back from the apex and becomes less evident.

Among the histochemical tests employed, it is easiest to explain the results of staining for RNA. As mentioned previously, there is conclusive evidence that this substance plays an active and vital role in protein
synthesis, giving it obvious importance in embryonic development.

During the morphogenesis of the scales, RNA is found primarily in the basal layer of epidermal cells, especially just above the basement membrane. Preferential localization in scale areas as opposed to non-scale areas is to be expected, since scale outgrowth obviously requires a greater rate of protein synthesis. Regional localization within the scale area is more difficult to explain. Accumulation primarily above the basement membrane might be related to the presence of a chemical substance, either just above or just below the membrane, which is necessary for the synthesis, maintenance, or activity of RNA. The appearance of RNA in the periderm in later stages could be related to the formation of keratin. It is more likely, however, that keratin has been synthesized at earlier stages in the basal epidermal layers and has migrated to the periderm (Wessells, 1961a). It is possible that synthesis of a "cementing" protein (Rogers 1959; Wessells 1961a) occurs in the periderm, in preparation for the cornification process.

Alkaline phosphatase (AP) appears to be closely related to RNA during the formation of scales, at least in a positional sense. It will be recalled that AP activity is found in the dermis of the developing scale, a high concentration being coincident with the dermal condensation. The AP is present extracellularly and intracellularly: extracellularly the enzyme has a fibrous appearance and may be adsorbed onto collagen fibers (Gold and Gould 1951); intracellularly, it is found in both nucleus and cytoplasm. The most striking fact is that the greatest activity of the enzyme is localized, in most stages, in a dark band
directly under the basement membrane. This localization suggests a transport function for the enzyme involving passage of materials from dermis to epidermis, and may or may not indicate a direct relationship between AP and RNA.

A transport function for AP was suggested by Hardy (1952) and by Danielli (1953), who proposed the detailed mechanism previously discussed. Moog (1946) believed that the enzyme, at least in kidney tubules, was involved in the phosphorylation of certain molecules as an aid to transport. It is possible that the phosphatase plays such a role in scale development as an aid in the transport of materials across the basement membrane for use in a particular metabolic reaction. Since AP and RNA are found on opposite sides of the basement membrane, it is likely that any functional relationship between the two substances is tied up with a transport mechanism, in which a material substance is carried across the membrane. Various hypotheses have been proposed to relate the enzyme with RNA through such a transport mechanism. Gibbey (1959) believed that AP is involved in the liberation of substances utilizable by RNA in protein synthesis. Koning (1957) suggested that the enzyme is involved in the transport of nutrients for use in the synthetic mechanisms of the epidermis. A direct role of AP in the construction of ribonucleoprotein was proposed by Hamilton and Koning (1956), who suggested that the enzyme splits phosphate esters and transports certain products across the basement membrane for use in RNA synthesis. Kischer (1960) proposed the alternate breakdown and resynthesis of transfer and possibly ribosomal RNA by the enzyme, as a necessary process of
differentiation. Whether AP is able to actively synthesize RNA (Krugelis 1946) is questionable, but if the process occurs in this system, the RNA precursors must be synthesized in the dermis, then transported in some manner to the epidermis for construction of RNA, since the greatest concentrations of AP and RNA are found on opposite sides of the membrane. Any one of these transport processes might conceivably link AP and RNA in the differentiation of tissues. It seems quite likely that the phosphatase may be involved in the transfer of either energy-providing substances or precursors needed for the formation of ribonucleoprotein. Considering the large number of steps involved in all well-known metabolic pathways, it is also likely that other enzymes are involved in the process, and that AP plays an important, but not solitary, role in such a system.

In spite of the apparent close relationship between AP and RNA, it is possible that such an association is primarily positional rather than functional. Since the basal epidermal region is apparently an area of rapid metabolism, and since high activity would involve a variety of chemical reactions, the enzyme might function in any number of ways, either directly or indirectly related to RNA activity. Fell and Danielli (1943) believed the enzyme to be necessary for the formation of structural proteins, a function which might be of some significance in this case. According to Chèvremont and Firket (1953) the AP of the nucleus plays an essential role in mitosis. Although the enzyme is found in the region of the scale with the highest mitotic rate, the very high activity of the enzyme in these areas implies a more extensive role. Before the role of
AP in differentiating tissues can be positively determined, further studies, especially in vitro, are necessary.

The presence of high concentrations of proteins in the epidermis, and especially in scale areas, is to be expected for several reasons. In the first place, RNA is generally found in a ribonucleoprotein complex, which would explain the high protein concentration just above the basement membrane, in association with the RNA. Secondly, the large amount of RNA in the epidermis of scale areas suggests that rapid synthesis of protein is occurring in these regions. Within the scale itself, protein seems to be in heaviest concentration on the flat surface, suggesting that keratinization occurs here either earlier or to a greater extent than in other areas of the scale.

The studies by Wessells (1961a) on development of chick leg skin in vitro correlate well with the results of the staining for general protein and for keratin. According to Wessells, kerato-hyalin granules appear in the primary and secondary periderm before scale formation begins, the process of keratinization being completed by the 15th day. This would explain the high protein and keratin concentration found in the periderm throughout the development of the scales. Wessells also pointed out that keratin tonofibrils are first seen in the lower halves of the basal epidermal cells, where the highest concentration of RNA is found. This suggests that keratin precursors, and indeed keratin itself, is formed primarily in the basal layers, from which region it is transported to the peridermal layers.

Since it is apparent that the basal epidermal layer is probably the
most active area in the growing scale, it is not surprising that a large number of mitochondria should accumulate in this region. The greatest concentration of the mitochondria is just above the basement membrane, where the RNA concentration is also highest. As was pointed out previously, the large amounts of RNA indicate rapid protein synthesis, which in turn suggests an area of high metabolic activity. Since the mitochondria of chick embryonic tissues have been shown to contain the enzymes of the Krebs cycle (Carey and Greville 1959b), this suggests that the mitochondria of the basement membrane permit a rapid carbohydrate metabolism, releasing large amounts of energy to be used in the synthesis of protein by RNA. The intimacy of association of RNA and mitochondria is questioned by Kischer (1962), and the histochemical results suggest that the association is functional rather than morphological.*

The study of mucopolysaccharides, using both the metachromatic reaction of toluidine blue and the PAS method, indicate the presence of two, and possibly three, distinct mucopolysaccharides. Metachromasia, or acid mucopolysaccharide, is found in the dermis, and especially at the basement membrane, as a fibrillar network. Michaelis (1944) believed this material to be a substance such as chondroitin sulfate, while Sylvén (1950) suggested that it consisted of labile sulfur-containing groups involved in keratin synthesis. In the developing scale, the metachromasia changes very little during the five days which were studied, suggesting that the acid mucopolysaccharide might play a predominantly structural role.

*According to Hamilton and Kischer (1961), RNA is not necessarily an essential structural element of the mitochondrion, but is a probable component of its activity.
role.

In PAS staining for neutral mucopolysaccharides a similar dermal network of positive material is found, distributed throughout the dermis and concentrated at the basement membrane. The material does not contain glycogen, but whether or not it is the same as the metachromasia could not be determined with the techniques used.

A positive reaction for glycogen is found in the periderm of the developing scales, in the form of fairly large globules of PAS-positive material which are removable by diastase. The number and size of the glycogen globules increase as development progresses, and in later stages the globules are located preferentially in the apex and inturned areas of the scale. The suggestion of Bradfield (1951) and of Wislocki and Dempsey (1945), that glycogen is found in active regions having only a limited blood supply, seems quite applicable in this case. The periderm is quite active in carrying out the process of keratinization at these stages, yet, being the outermost layer of the non-vascular epidermis, the periderm probably receives little sustenance by diffusion from the blood. Thus the glycogen might act as an energy source for the maintenance of the cells of this layer, in which anaerobic oxidations might be necessary. If, as was suggested previously, the flat surface of the scale completes keratinization before other regions, this would explain selective localization of keratin at the turned-in area in later stages.

In spite of suggestions by other investigators that alkaline phosphatase and glycogen are intimately related during certain developmental processes, no evidence was found in this study that such a relationship
is important in morphogenesis of scales. The peak of glycogen concentra-
tion not only appears later than that of AP, but is found relatively far
removed from sites of greatest enzymatic activity.

Comparison of differentiation in scales and down feathers reveals
many morphological and histochemical similarities. In the earliest
stages the primordia of the two organs appear to be morphologically
identical. Thus an early down feather of approximately the eighth day of
incubation, consisting of a condensation of dermal cells covered by a
thickened epidermis, is essentially indistinguishable from a scale from
an 11-day embryo. On the next day of incubation the two organs (9-day
feather and 12-day scale) are also quite similar, even to the extent that
the outgrowths begin bending in a particular direction. After another
day of development, however, marked differences become apparent. While
the scale begins overlapping and develops a flat surface, the down
feather continues rapid outgrowth, becoming quite elongated.

Histochemically the two organs are also very similar in early stages.
Both have intense cytoplasmic basophilia, shown to be RNA, in the basal
epidermal cells of thickened areas. Even after several days of develop-
ment, both organs still show a strong reaction for RNA at the basement
membrane.

Alkaline phosphatase is present in the dermal condensation of the
early feather germ, as in the young scale, and is concentrated at the
apex of the down feather. In older feathers, the highest activity is
found at the base of the germ, especially in dermal areas adjacent to the
epidermis. Such proximity to the dermis is found in the development of
scales, where activity of phosphatase is greatest under the basement membrane. In the scale, however, activity is high throughout the dermis of scale areas, rather than being localized, as it is in the base of the feather germ.

Staining for general proteins in the developing down feather indicates the greatest concentration to be in the epidermis of the germ; this is also true of the forming scale. Although specific keratin tests were not made on the down feather it is probable that keratinization is completed relatively later in the feather than in the scale, since early keratinization of the feather would interfere with further outgrowth.

Acid mucopolysaccharides in the feather germ are most evident in the pulp in the form of a fibrillar matrix, similar to the fibrillar matrix found in the dermis of the developing scale. Neutral mucopolysaccharides are faintly stained in the pulp of the feather, but apparently are not distributed in a dermal network as in the scale.

Glycogen is fairly abundant in the cuticle of the feather and in the entire skin; the strongest reaction, however, is in the centers of the barb ridges. The glycogen of the barb ridges appears to be similar to that found in the periderm of the older scales, as both are in the form of globules. Such globules, however, apparently do not form in the cuticle of the down feather.

Thus, comparisons between feather and scale development show that, at least for the first two days, the process is nearly identical for both. At the end of this time distinct differences in histochemistry and morphology become evident. The reasons for such diversification in
developmental pathways are not known, but further considerations of the problem are presented below.

The relationship between biochemical processes and inductive specificity in developing embryonic systems has been the basic question in several recent studies. As early as 1947, Brachet found that ribonucleoproteins are a necessary component of any inductor or evocator. Since this time, various investigators have, by their experiments, implicated either the protein or the RNA portion of the ribonucleoprotein as the inductor; the most recent investigations by Lash (1962), however, present strong evidence that a nucleotide fraction of this material is the active agent in induction. In relating such information to the development of integumentary derivatives several difficulties are encountered. Rather than finding RNA in highest concentration in the inducing tissue, the dermis, it is found to be accumulated in the basal region of the reacting tissue, or epidermis. A possible answer to this discrepancy is that the induction actually occurred previous to the earliest stage used in the histochemical study. As McKeehan (1956) observed, the highest concentration of RNA seemed to pass from the retina to the overlying ectoderm during lens induction in the chick. A similar process could be in operation during induction of scales in the chick; that is, the RNA, or ability for rapid RNA synthesis, might pass from the dermis to the basal cells of the epidermis during induction, thereby explaining the high concentration above the basement membrane in later stages.

The relationship of RNA to specificity also presents some interest-
ing possibilities. It is evident that regional specificity is directly dependent on RNA, since this material determines the type of protein which will be synthesized. For example, the rate and quantity of keratin synthesis during feather and scale development would depend on the RNA present. If it is assumed that the dermis is the primary inductor, and that induction is due to the RNA of the dermis, then it does not seem too unlikely that the dermal RNA is also involved in the specificity of the integumentary derivatives. If specificity is determined by the dermal layer, then the high concentration of RNA in the epidermis, which is believed to be involved in synthesis of keratin and other proteins, must be directed in some manner by the dermis. This subject is considered further in the general conclusions.

If alkaline phosphatase is indeed related to RNA, as is suggested by various studies, its relationship to inductive specificity provides another interesting possibility for future research. The implication by Moss et al. (1961) that alkaline phosphatase might be characteristic of the tissue from which it is extracted suggests that the enzyme could be related to specificity in embryonic development. To test this idea, comparison of phosphatases from various embryonic tissues rich in the enzyme would be necessary. However, until the possible relationship of RNA to phosphatase is elucidated, the significance of such an experiment would be unclear.
GENERAL CONCLUSIONS

Having discussed both parts of this investigation, an attempt will now be made to relate the conclusions drawn in the two areas. In the histochemical study, it was suggested that RNA is the active inductive agent in the development of integumentary derivatives and that this same substance is directly responsible for specificity. Based on the work of McKeehan (1956) it was also pointed out that heavy concentrations of RNA might be found in the epidermis only after induction has occurred.

The results of the grafting experiments and the results of experiments of other investigators suggest that the dermis is specific from the time of its origin, but that this layer must be evoked by the deeper tissue before dermal inductivity can manifest itself.

Relating the results of the two parts of the investigation the following mechanism is suggested: the dermis might be capable of producing only a specific type of RNA - that which will direct development towards a specific integumentary differentiation; the dermal RNA is probably brought into action only when a necessary non-specific substance is provided by the differentiating deeper tissue. After this "activation" or synthesis of dermal RNA, induction of the epidermis could occur, presumably through the RNA of the dermis. The presence of large quantities of RNA in the epidermis during development of the integumentary derivatives is probably the result of a rapid synthesis directed in some manner by the dermis. After induction by the dermis and synthesis of RNA in the epidermis, development of the specific integumentary
derivatives could take place.

In regard to the question of bipotentiality of leg skin, little can be said, because the mechanism is not well understood. It is possible, however, that leg dermis, under proper conditions, is capable of producing RNA specific for both scales and feathers.

A few words should be said regarding the genetic implications of these hypotheses. Since the type of RNA produced depends on the DNA present in the nuclei, and thus on the genetic makeup of the organism, then all of these mechanisms must depend ultimately on the genetic background. The relationships of genetics to differentiation, however, and especially the mechanisms of differential gene activation, are beyond the scope of this dissertation. It may simply be said that certain genes act at different times during the various developmental processes, so that only certain types of RNA are produced at a given time and in a given tissue. In regard to integumentary derivatives, an interesting example of genetic effects is found in certain strains of chickens which have feathers on their legs in normally scaled areas.

The problem of the development of integumentary derivatives is also of interest to the evolutionist, since feathers are believed to have evolved from scales. This appears quite likely on the basis of the similarity of the two types of derivatives in the early stages of development, both histochemically and morphologically. It is probable that the biochemical pattern of scale development is evolutionarily more primitive than that of feather development.

Thus, although much work has been devoted to the elucidation of
this complex developmental system, further research on the biochemical and embryonic phenomena involved is needed before the entire process can be known with certainty.
1. Grafts were prepared using combinations of skin and deep tissue and were grown for 7 or 8 days on the chorioallantoic membrane. The grafts fell into three main groups: those using indifferent "feather skin," those with indifferent "scale skin," and those using skin which was in the process of forming scales.

2. Grafts with indifferent "feather skin" plus deep leg tissue formed feathers in all cases, indicating that the feather dermis is unaffected by changing the substrate.

3. Grafts made with indifferent "scale skin" plus various deep tissues formed all scales, showing that the deep tissue had no apparent effect on dermal specificity.

4. When skin in which scales were forming was used, only scales formed on the grafts in most cases; however, in a few instances in which deep wing tissue was used, a few feathers developed on the grafts. This suggests that leg skin is bipotential.

5. On the basis of the results of these experiments and on the basis of the pertinent literature, it is suggested that the dermis is innately specific, but that deep tissue is necessary for the manifestation of the inductive specificity of the dermis.

6. The possibility of bipotentiality of leg skin is discussed, with speculations on the mechanism involved.

7. Sections were cut from the legs of chick embryos which had been incubated for 10 to 14 days. The sections were treated with histochemical procedures for demonstrating ribonucleic acid, alkaline
phosphatase, protein, mitochondria, and mucopolysaccharides.

8. Ribonucleic acid was localized preferentially in scale-forming areas, especially just above the basement membrane. Such a localization suggests that rapid synthesis of proteins occurs in the basal epidermal cells.

9. Alkaline phosphatase was found in the dermal region of the developing scale, and was in greatest concentration just under the basement membrane. This suggests that the enzyme is involved in transport across the membrane. Such a transport mechanism may be involved with either the synthesis of RNA itself, or the synthesis of protein by RNA.

10. Protein concentrations were heaviest in the epidermis, both at the basement membrane and in the periderm. The concentration at the basement membrane was probably due to the protein of the ribonucleoprotein complex and to the protein being synthesized by the RNA. In the periderm the protein which accumulated was presumably keratin or keratin precursors.

11. Mitochondria were abundant in the basal epidermal cells, especially in areas rich in RNA. Since the area above the basement membrane is an area of active synthesis, and therefore requires a continual source of energy, such energy might be supplied through the metabolic reactions of the mitochondria.

12. Glycogen was found mainly in the periderm, especially in later stages of development of the scale. It is believed that glycogen might be needed in this area as a stored energy source, since the
periderm is farthest removed from the blood supply.

13. A comparison was made between the histochemistry and morphology of developing scales and down feathers. It was found that development of the two derivatives was similar during the first two days, after which time differences became pronounced.

14. The possible functions and interrelationships of the various chemical substances and the problem of inductive specificity are discussed.
LITERATURE CITED


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Plate I

Sections stained with toluidine blue

Figure 1. Sagittal section of leg of an embryo of stage 36, showing a scale area. The epidermis is thickened, and basophilia is concentrated in the periderm and just above the basement membrane. X400

Figure 2. Section similar to that in Figure 1, but treated with ribonuclease before staining. The spaces in the periderm and above the basement membrane indicate the presence of RNA in these areas. X400

Figure 3. Sagittal section of leg of an embryo of stage 37, showing two young scales. Note the condensation of cells in the dermis of scale areas. Basophilia is heaviest in scale areas, especially above the basement membrane. X160

Figure 4. Control section for Figure 3 of an embryo of stage 37-, treated with ribonuclease. The clear spaces in the periderm and basal epidermal cells show the areas of high RNA concentration. X160

Figure 5. Sagittal section of leg of an embryo of stage 39. Note the dark line of basophilia above the basement membrane. X160

Figure 6. Control section for Figure 5, treated with ribonuclease. The clear line above the basement membrane, and the clear spaces in the periderm indicate the presence of RNA. X160
Plate II

Sections stained with toluidine blue

Figure 7. Sagittal section of leg of an embryo of stage 33. A dark basophilic border is present below the nuclei of the basal epidermal cells. Note the condensation of dermal cells in the scale apex. X400

Figure 8. Control section for Figure 7, treated with ribonuclease. Clear areas in the epidermis, most apparent above the basement membrane, indicate high concentrations of RNA. Fibrillar network in the dermis is metachromasia. X400

Figure 9. Higher magnification of inturnd area of scale shown in Figure 7, showing heavy basophilia concentration above the basement membrane. X1500
Plate III

Sections stained by Gomori method

Figure 10. Sagittal section of leg of an embryo of stage 37-, showing an early scale. Alkaline phosphatase activity is found throughout the dermis, but is slightly more concentrated in the scale area. X160

Figure 11. Control section for Figure 10, incubated in a mixture lacking glycerophosphate. Note complete lack of phosphatase staining. X160

Figure 12. Sagittal section of leg of an embryo of stage 38. Phosphatase is concentrated throughout the dermis of the scale and is found in a dark line just under the basement membrane. X160

Figure 13. Section cut through a scale of an embryo of stage 39-, showing high phosphatase activity both intracellularly and extracellularly. X400

Figure 14. Control section from leg of an embryo of stage 39, incubated in a mixture lacking only the glycerophosphate. Phosphatase staining is completely lacking. X400

Figure 15. Sagittal section of scale of an embryo of stage 40. Note high phosphatase activity in all of the dermis, especially in the scale apex and under the basement membrane. X160
Plate IV

Sections stained with mercuric-bromphenol blue

Figure 16. Sagittal section of leg of embryo of stage 37, showing two developing scales. Protein concentration is heaviest in the periderm and above the basement membrane. Dermal cells are concentrated in scale areas. X160

Figure 17. Section of scale of an embryo of stage 38, showing heavy protein concentration in the epidermis and condensation of dermal cells in the apex of the scale. X400

Figure 18. Sagittal section of a scale from an embryo of stage 39. Condensation of dermal cells has moved back from the apex and is found above the inturned area. X160

Figure 19. Higher magnification of scale of Figure 18. Note layers of cells in epidermis and high concentration of protein in the periderm and just above the basement membrane. X400

Figure 20. Section of leg of an embryo of stage 40, showing overlapping scales. The smooth outer border of the periderm, as well as the alignment of nuclei in this layer, indicates that keratinization has occurred at this stage. X160
Plate V

Sections stained with Regaud's method

Figure 21. Scale area of an embryo of stage 36, stained with iron haematoxylin. Mitochondria are found in the basal layer of epidermal cells, in highest concentration above the basement membrane. X1500

Figure 22. Section of a scale of an embryo of stage 37-. Mitochondria are found in the basal epidermal cells, surrounding the nuclei, with the greatest number just above the basement membrane. Note the elongated shape of the mitochondria. X1500

Figure 23. Scale area of an embryo of stage 39, showing part of the apex and part of the inturned area. Distribution of mitochondria is similar to that of earlier stages, but the mitochondria seem to be shorter than in earlier stages. X1500
Plate VI

Sections stained with periodic acid-Schiff method

Figure 24. Section of leg of an embryo of stage 36, showing an early scale. Fibrillar network in dermis, dark line at the basement membrane, and dark material in periderm indicate neutral mucopolysaccharides. X400

Figure 25. Control section for Figure 24, treated with diastase before staining. Lack of a staining reaction in the periderm indicates the presence of glycogen in this area. X400

Figure 26. Section of scale of an embryo of stage 38. Dark-staining material in the periderm is found mainly at the base and inturned area of the scale. X160

Figure 27. Diastase-treated control for Figure 26, showing lack of positive reaction in the periderm. X160

Figure 28. Scale from an embryo of stage 39, showing dark globules of PAS-positive material in the periderm, especially in the inturned area. X400

Figure 29. Control for Figure 28, treated with diastase. Lack of positive reaction in the periderm identifies the globular material of Figure 28 as glycogen. X400

Figure 30. Section of a scale of an embryo of stage 40. Periderm stains darkly, especially in the inturned area. Note also the distribution of the dermal fibrillar network. X160

Figure 31. Control section for Figure 30, treated with diastase. Loss of PAS-positive material from the periderm indicates that this substance is glycogen. The fibrillar network, however, is apparently unaffected by the enzyme. X160