INTRODUCTION

In earlier work it was found that the addition of excess of vitamin A (10 i.u./ml.) to the culture medium produces drastic changes in embryonic limb-bones and skin in organ culture. The matrix of cartilage (mouse, chick) and bone (mouse) rapidly disappears (Fell & Mellanby, 1952; Fell, Mellanby, & Pelc, 1956) and in the epidermis of skin from 7 to 18-day chick embryos (Fell & Mellanby, 1953; Fell, 1957; Pelc & Fell, 1960) keratinization is immediately arrested and a remarkable mucous metaplasia appears.

In a previous paper (Fell & Thomas, 1961) it was shown that when hydrocortisone (7.5 μg./ml.) is added together with vitamin A, resorption of intercellular material is greatly retarded in the explanted limb-bone rudiments. The present experiments were undertaken to see whether the hormone would also inhibit the metaplastic effect produced by vitamin A in the epidermis of embryonic chicken skin in vitro. A mutual antagonism between the two agents in their action on the epidermis has been demonstrated, and is described below.

MATERIAL AND METHODS

Material

Skin was stripped from the back and sides of 9-day embryos and cut into fragments about 3 mm. in diameter.

Culture methods

The explants were grown by Shaffer’s modification (Schaffer, 1956) of the watch-glass (moist chamber) method of Fell & Robison (1929).

In each culture vessel a clot was made in the watch-glass by mixing 15 drops of cock’s plasma with 5 drops of embryo extract. The extract was prepared by adding Tyrode’s solution containing 1 per cent. (w/v) glucose to an equal volume

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of the finely ground pulp of a 13-day chick embryo, and centrifuging the mixture for 5 minutes. Each skin fragment was spread on a square of rayon acetate cloth with the epidermis upwards, and 4 squares were laid on the surface of each clot. Every 2 days the squares were lifted from the clot, washed first in Tyrode’s solution and then in dilute embryo extract made without extra glucose, drained, and transferred to fresh medium. For further details of the procedure see Fell (1957).

Addition of hydrocortisone and vitamin A to the medium

Hydrocortisone sodium succinate (Solu-cortef, Upjohn Co., Kalamazoo) was dissolved in sterile distilled water and added to the plasma so as to give a concentration of 7.5 µg./ml. in the final medium.

Synthetic vitamin A alcohol (Roche Products Ltd.) was dissolved in ethanol and added to the plasma to give a concentration of either 10 or 5 i.u./ml. in the final clot. (See Fell & Thomas, 1960.)

The different media whose effects were to be compared all contained the same quantity of water and/or ethanol.

Design of experiments

The designs of the various experiments and the number of explants used in each are shown in tables 1 and 2.

Histology

The explants were fixed in Zenker’s fluid + 3 per cent. glacial acetic acid for 30 minutes, then immersed in Zenker’s fluid without acetic acid for a further 1½ hours. In exp. 310 (Table 1) the explants were fixed for 15 minutes in acetic acid alcohol (1:3) followed by 45 minutes in formal saline (cf. Pelc & Fell, 1960); this gave excellent preservation of the viscid secretion mentioned below. After being washed in tap-water and dehydrated, the explants were placed in 3 successive baths of acetone which dissolved the rayon cloth from the tissue, after which they were cleared in 3 successive baths of cedar-wood oil before being embedded in paraffin wax.

Serial sections were cut at a thickness of 7 µ and stained by the following methods: azan; the periodic acid/Schiff method (PAS), with and without diastase digestion, followed by Mayer’s acid haemalum; Mayer’s acid haemalum and alcian blue for the demonstration of mucin.

RESULTS (Table 3)

Normal 9-day skin and feather germs

Chick embryos of the same age vary somewhat in their degree of development. There is also a regional variation in the skin in the same embryo, differentiation being farthest advanced near the mid-dorsal region of the trunk and on the outer surfaces of the proximal part of the limbs.
Skin

In the trunk the epidermis (Plate 2, fig. 10) consists of a basal layer of columnar epidermal cells, and a periderm composed of a very thin upper and an incomplete lower layer; the upper layer secretes material that stains with alcian blue. The epithelium rests on a basement membrane below which is a loose dermis consisting of spindle-shaped or stellate cells oriented roughly parallel with the epidermis, and a fine network of intercellular fibres.

Feathers

Feather rudiments (Plate 1, fig. 1) are present. The youngest consist only of a dense rounded condensation of mesoderm underlying an area of slightly thickened epithelium, while the most highly developed appear as ovoid projections. They are not yet pigmented and follicles have not begun to form.

The epidermis of the feathers is further differentiated than that of the skin proper. Both layers of the periderm are distinct and the epidermal cells have more strongly basophilic cytoplasm and are more compactly arranged than in the skin epidermis. In the larger rudiments there may be 3–4 layers of epidermal cells near the tip where development is always most advanced. The mesoderm of the future pulp is very compact in all the feather germs.

Control explants in normal medium

(Table 1, exps. 301, 305, 334; Table 2, exp. 341)

<table>
<thead>
<tr>
<th>No. exp</th>
<th>Culture medium</th>
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<tr>
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<td></td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC+A</td>
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<tr>
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<td>Normal</td>
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<td></td>
</tr>
<tr>
<td></td>
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<tr>
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<td>Vit. A 5 i.u./ml.</td>
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<tr>
<td></td>
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<td>HC+A</td>
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</table>

HC = hydrocortisone; A = vitamin A; HC+A = both agents added together

Twenty-three explants were grown in normal medium for 1–10 days.

Skin

The epidermis differentiated rapidly, the peripheral zone of the explant being always the most, and the central region the least, advanced. At 2 days the
periderm was two-layered and contained some alcian blue staining material which was apparently being secreted. By the 4th day, in places the lower layer of the periderm had become filled with cytoplasmic granules which, like keratin, stained a deep orange with azan; this appearance, which is also characteristic of the normal periderm in vivo, was most common near the margin of the explant but occurred in patches elsewhere. The epidermis was thicker than at explantation and an intermediate layer had been formed; near the edge of the skin a very thin layer of keratin was sometimes present beneath the granular cells of the periderm, and could be seen in life as a fibrous ring round the explant.

After 6 days the epidermis usually comprised cubical basal cells, 2 layers of prickle cells, about 3 flattened layers corresponding to the mammalian stratum granulosum, and a thin stratum corneum beneath the now degenerate periderm which was being sloughed; the keratin had become fairly thick by the 8th (Plate 2, fig. 11) and 10th days. As found in earlier work (Miszurski, 1937; Fell & Mellanby, 1953) keratinization was precocious in the cultures as compared with normal skin of comparable age; thus in an 18-day chick a stratum corneum was not yet present, although the lower peridermal cells were stuffed with cytoplasmic granules like those described above.

The dermis became denser and more fibrous during cultivation but otherwise changed little.

Feathers

In the living cultures the feather rudiments elongated during the first 4 days and became rather narrow at the base and pointed at the ends; new papillae also appeared. After this stage growth ceased, the keratinizing rudiments became more opaque and sometimes the ends of the longer feather germs were slightly distended. In some feathers slight pigmentation was seen as early as the 2nd day, many were pigmented by the 4th day, and the blackening increased slightly during subsequent cultivation.

Histological examination at 2 days (Plate 1, fig. 2) showed that in explants from the more advanced embryos, the epidermis of the larger feather germs had begun to differentiate into a multilayered sheath of flattened cells enclosing thick infoldings that normally would form the barb vanes; the follicles also had begun to appear as a small invagination round the base of the feather. These developmental processes were well advanced in all explants by the 4th day. As in normal development (Goff, 1949), the narrow base of the feather, or umbilicus, was filled with a dense mass of fibrous tissue while elsewhere the compact mesoderm had changed into a loose stellate reticulum which was very sparse near the tip of the rudiment. The feather had now become sunk into a follicle of fairly normal appearance, and slanted at an angle of about 45° to the surface of the skin.

After 6 days the sheath had begun to keratinize, and differentiation of the germ then stopped, probably owing to starvation of the enclosed cells. Some of
the fibroblasts and epithelial cells in the tip of the feather degenerated, but others retracted from the keratinized sheath to form part of a viable papilla in the base of the otherwise hollow rudiment. This papilla persisted, and by the 8th or 10th day formed a compact mass of cells and fibres sometimes covered by a layer of the retracted epithelium which thus formed a pulp cap, separating the dermal papilla from the cavity of the feather as *in vivo* (cf. Goff, 1949).

*The effect of hydrocortisone alone*

(Table 1, exps. 301, 305, 334, 335)

Twenty-seven explants were grown in medium to which only hydrocortisone had been added.

*Skin*

After 2 days' growth, the surface of the epithelium in life was covered with small transparent globules which in section proved to be the enormously dilated cells of the periderm. In spite of this swelling, the lower peridermal cells contained the characteristic cytoplasmic granules mentioned above, which in the controls did not appear before the 4th day. Sometimes these granules were present in large numbers, so that the cells were stuffed with them. The superficial cells of the periderm stained strongly with alcian blue and a layer of blue-staining material adhered to the surface. In one explant a different type of secretion was present in one area; it had a homogeneous appearance, stained a deep reddish-orange with azan like keratin but unlike mucin, and was extremely hard to section. It was being ingested by macrophages which were stuffed with globules of the material.

The differentiation of the epidermis was several days in advance of that in controls grown in normal medium; at 2 days it consisted of closely packed cuboidal basal cells with abundant, deeply staining cytoplasm, a single spinous layer, and one or more superficial flattened layers immediately beneath the periderm. In the single explant fixed at 4 days, a thin stratum corneum was already present.

By the 6th day most of the periderm had been shed, and after 8–10 days a much thicker layer of keratin had been formed than in the corresponding controls (cf. Plate 2, figs. 11, 12).

*Feathers*

The gross anatomical development of the feathers was arrested at a much earlier stage than in the controls; this was well seen in the living cultures. The effect of the hormone depended on the degree of development when treatment began. The youngest papillae did not elongate but formed a broad circular structure just below the surface of the epidermis. Slightly older rudiments produced a broad, round base from the centre of which a small knob or cone projected; the better-developed feather germs gave rise to thin sausage-shaped
structures, the ends of which sometimes became distended into a balloon. Pigmentation was poor and the pigment cells were usually rounded bodies very different in appearance from the fine, branched melanophores of the controls.

Histological examination showed that this early abortion of the feathers was associated with a precocious differentiation of their epidermis. Already at 2 days (Plate 1, fig. 3) the feathers were sunk into follicles, and the epidermis had differentiated into sheath and deeply folded barb-vane rudiments; mitosis was very abundant in the basal cells (stratum cylindricum). Even the small papillae had differentiated into sheath and barb-vane ridges, and acquired a follicle. The epithelium of the young papillae was embedded in a sharply defined rounded mass of dense mesoderm. In the older rudiments the pulp tissue had partly withdrawn from the tip of the feather but was very condensed in the proximal part. As in the skin, the peridermal cells were enormously dilated.

By the 4th day the epidermal sheath had begun to keratinize. The keratin increased with age, but otherwise the feather germs remained almost unchanged.

*The effect of vitamin A alone*

(Table 1, exps. 296, 305, 334, 310, 335; Table 2, exp. 307)

Twenty-two explants were grown for 1–10 days in medium to which had been added 10 i.u. vitamin A/ml. (exps. 296, 305, 334, 307) and 20 in the presence of 5 i.u./ml. of added vitamin (exps. 310, 335).

*Skin*

In the living cultures, the explants differed from those in normal and hydrocortisone-containing medium by failing to keratinize and remaining translucent throughout the culture period. By the 6th or 8th day, mucus could usually be seen with the naked eye as long, clear strands stretching between the explant and the clot when the skin was lifted from the watch-glass for transfer or fixation.

Histological examination showed only minor differences between the effects of the two concentrations of vitamin A, and the two series will therefore be described together.

The periderm developed differently from that of either the control or hydrocortisone-treated explants, and behaved as an integral part of the epithelium, probably owing to the inhibition of keratinization. Since mitosis remained active not only in the peridermal elements but also in the epidermal cells at all levels, it was sometimes almost impossible to tell where a given cell had originated.

After 4 days, in many areas the superficial peridermal cells had become arranged as a regular cuboidal epithelium resting on a lower layer of ovoid cells; mucus was being secreted by the upper cells many of which had developed fine processes resembling cilia. These cilia-like processes were particularly well seen in the 6-day cultures of exp. 335 (Plate 3, fig. 15) which had been exposed to
the lower dose of vitamin. Cytoplasmic granules like those of the control and hydrocortisone-treated explants could not be detected in the periderm by the light microscope.

Below the periderm the cells showed little change during the first 6 days, but the epithelium became considerably thicker in the cultures grown in the presence of 5 i.u. vitamin A/ml. than in those treated with the higher concentration owing to the larger size of the cells and better development of the intermediate layer. By 8 days mucous secretion was fairly profuse with the higher dose (Plate 2, figs. 13, 14) and while in places the epithelium retained its stratified structure, in some areas, especially near the margin of the explant, the outer cells had been shed and the epidermis consisted of an orderly layer of columnar cells resting on a cuboidal basal layer.

This thinning of the epithelium at the higher concentration progressed, and at 10 days stratified epithelium was usually present only in the less-differentiated central areas. Most of the periderm had been sloughed, and many of the cells now at the surface were pigmented; pigment granules and rods were sometimes being extruded with the mucin which at this stage was plentiful in nearly all the superficial cells. In one culture a tidy row of fully formed goblet cells rested on a single shallow basal layer. Cells with cilia-like processes were rare at this stage.

With the lower dose, the stratified structure of the epithelium persisted to the 10th day (Plate 3, fig. 16). Although mucin was present in cells at all levels (Plate 3, fig. 17) and goblet cells were common, the total amount of secretion was rather less than with the higher concentration. Large tracts of the outermost layer resembled the original periderm. Mitoses were present throughout the epithelium including the superficial cells, and were more abundant than with the larger concentration of vitamin.

**Feathers**

In the living cultures, pigmentation of the feathers appeared earlier and was more intense than in the controls. The feather germs enlarged somewhat, failed to keratinize, and the ends retained their rounded appearance, thus differing from the pointed ends of the controls in normal medium. During the later stages of cultivation the bases became very constricted, and many of the feathers were detached. The feathers did not become distended, but the pulp retracted from the distal epidermis though less than in the controls.

On histological examination the structure of the feathers was found to change little during the first few days in culture. In the explants grown in the presence of 10 i.u. vitamin A/ml., a sheath was not formed, but at the lower dose one differentiated though to a lesser degree than in the controls. The vitamin-A-treated feathers were flabby and often partly rested on the surface of the skin (Plate 1, fig. 4) with which they sometimes fused; similarly, feather germs in close proximity usually fused laterally.

The follicular invagination, instead of dipping into the dermis at an angle of
about 45° as in the controls, proceeded almost parallel with the surface of the skin to form an annulus which gradually cut across the base of the rudiment; this accounted for the basal constriction seen in the living cultures, which often lead to the detachment of the rudiment as described above.

Where the pulp had retracted from the epidermis at the tip of the feather, the space between the two tissues was filled with material that stained intensely with alcian blue. This contrasted with the feebly staining coagulum that occupied the empty ends of the control and hydrocortisone-treated feather germs.

The epithelium covering the proximal part of the feather germs often became secretory like that of the skin, but there was little or no secretion from the distal surface. The follicle sometimes behaved almost like a gland with mucus being secreted into irregular cavities in the interior.

*The combined effects of vitamin A and hydrocortisone*

(Table 1, exps. 296, 305, 334, 310, 335)

Forty explants were exposed to both agents simultaneously.

**Skin**

The results showed that vitamin A and hydrocortisone were mutually antagonistic in their action on the skin, which resulted in a curious struggle for supremacy between the two agents.

Especially during the first 2 days in culture, a remarkable phenomenon was observed. As described above, in one area of an explant treated with hydrocortisone alone, the periderm secreted a material that like keratin stained a deep reddish-orange with azan. Under the joint influence of hydrocortisone and vitamin A, the periderm produced this substance on a large scale, so that in the majority of explants it formed a thick viscid layer over much of the surface; in the living cultures it was very refractile and after fixation it became extremely hard.

In section, the secretory periderm appeared as an orderly superficial row of cubical or columnar cells resting on a flattened layer beneath which was the epidermis. The secretion after digestion with diastase stained an intense reddish-orange with azan (Plate 1, fig. 6) and deep crimson with PAS. The secretory period was often brief, and when it ended the cells of both layers of the periderm became greatly distended and appeared nearly empty, as with hydrocortisone alone. In some explants this peridermal swelling had affected almost the whole surface of the skin by the end of the 2nd day, but in others secretion continued for several more days in some areas. By the 4th day the structure of the periderm often showed great regional variation in the same explant, the cells being swollen and vacuolated in some areas, mucus-secreting in others, and elsewhere producing the original azan-staining material which also stained weakly with alcian blue. With the lower dose of vitamin (5 i.u./ml.) a few cells with cilia-like processes were seen.
Meanwhile the epidermal cells proliferated and by the 4th day had usually formed a rather irregular stratified epithelium beneath the periderm. That the true epidermal cells also were able to secrete the peculiar material described above, was well shown by exp. 310 for which only 5 i.u. vitamin A/ml. had been used in conjunction with the hormone. In this experiment little secretion was present in the explants fixed after 2 days; both peridermal layers were greatly dilated, while the underlying epidermis comprised two layers of normal appearance. During the next 48 hours the epidermal cells multiplied actively to form a stratified epithelium composed of a basal stratum with plentiful mitosis, 1–2 layers corresponding to a stratum spinosum and one or more flattened layers. The flattened cells were thickly covered by secretion which, like the cells, stained a deep reddish-orange with azan (Plate 1, fig. 7) and crimson with PAS (Plate 3, fig. 19) after digestion with diastase. In sections stained with haematoxylin the necrotic remains of the periderm could be distinguished in places, embedded in the dense secretion.

After 6 days in culture nearly the whole surface of the explants in exp. 310 was keratinized, and the well-differentiated epidermis resembled that of an explant treated with hydrocortisone alone. In the other experiments, however, the structure of the epidermis, like that of the periderm at an earlier stage, usually varied widely in different areas of the same explant. In some regions the epithelium might be thick and well keratinized (Plate 4, fig. 20); elsewhere it might consist of 2–3 layers with a superficial stratum of flattened or cuboidal cells and no keratin (Plate 4, fig. 21). Whether these superficial cells were true epidermal elements or represented persistent periderm could not be determined; in some areas they had cilia-like processes and were secreting mucus as in the cultures exposed to vitamin A only, while nearby they were sometimes either secreting the viscid refractile material formed so profusely at 2 days, or producing a curious mixed secretion in which areas stained like mucus were mingled with material giving the staining reactions of the early viscid substance.

By the 8th or 10th day the vitamin had prevailed over the hormone. The keratin formed at a previous stage was being sloughed (Plate 4, fig. 22) and the epithelium contained mucus-secreting cells. Sometimes a thick layer of the early viscid secretion was separated from the epithelium by a broad zone of mucus, showing that the cells had changed their synthetic activities in response to the growing influence of the vitamin.

When the lower dose (5 i.u./ml.) of vitamin was used (exps. 310 & 335), the epidermis showed more cellular sloughing and damage at the end of the culture period than with the higher concentration.

**Feathers**

In the living cultures, the larger feather germs were seen to undergo a remarkable change. After only 24 hours they had swelled into thin-walled balloons, and by 48 hours they had made contact with each other laterally, and by mutual
pressure had acquired a polyhedral shape reminiscent of the bubbles of a soap lather. The small feather papillae failed to develop, as in the explants treated with hydrocortisone alone. In many explants, after 2 days the viscid secretion described above could be seen as clear glistening material above and between the feathers. As with hydrocortisone alone, pigmentation was severely inhibited. After 8–10 days many of the dilated feathers shrivelled and fell off, leaving only a small basal stump.

In section (Plate 1, fig. 5) the distal part of the swollen feather was empty, but the proximal half was filled with a sparse network of connective tissue. The balloon-shaped rudiment was attached to the skin by a short stalk containing dense mesoderm. The attenuated epidermis of the balloon was covered by periderm, the cells of which were sometimes swollen and nearly empty, and sometimes secreting the azan-staining material.

With the lower dose of vitamin A, the epidermis of the smaller papillae and that covering the stalk of the larger rudiments differentiated precociously into a thick sheath of flattened cells enclosing deep folds representing the barbs; it thus behaved like that of feather germs exposed to hydrocortisone alone. In explants grown in the higher concentration of vitamin A, only traces of this differentiation appeared.

The feathers underwent little further change except that with the lower dose of vitamin, some of them keratinized; this keratin was eventually sloughed from the small papillae.

The effect of transferring vitamin-A-treated explants to medium containing added hydrocortisone

Five explants (Table 2, exp. 307) were grown for 8 days in medium to which only vitamin A (10 i.u./ml.) had been added, and were then transferred for

<table>
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<tr>
<th>No. exp.</th>
<th>First culture medium</th>
<th>First culture period (days) and no. explants fixed (brackets)</th>
<th>Second culture medium</th>
<th>Second culture period (days) and no. explants fixed (brackets)</th>
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<td></td>
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<td>&quot;</td>
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<td>&quot;</td>
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HC, hydrocortisone.
be formed when the tissue was treated first with the vitamin and then with the hormone.

**Skin**

Two explants were fixed immediately after 8 days’ growth in the presence of vitamin A. The epidermis consisted of a non-keratinized stratified epithelium, 4–5 layers in depth and containing many mucus-secreting cells; mitosis was seen at all levels and there was much pigment. It was difficult to know whether periderm was present because, as stated above, in medium containing vitamin A it behaves as an integral part of the epidermis. The superficial cells did not become distended.

Two days after transfer to hydrocortisone-containing medium, large quantities of refractile secretion were visible on the surface of one explant and smaller amounts in others. In sections stained with azan (Plate 2, fig. 8) a superficial layer of reddish-orange-coloured material was seen, which appeared to be identical with that formed in the cultures treated with both agents at once except that it was associated with abundant mucus stained the usual pale blue; with alcian blue, the material that acquired a reddish colour with azan, stained feebly, while the mucus gave the typical brilliant blue reaction. Mucous secretion was most copious at the margin of the explant, and the other material was mainly formed in the central region where the metaplastic effect of the vitamin-A-pretreatment was least advanced. Whether the viscid secretion was produced wholly or partly by persistent periderm, was uncertain.

Four to six days after exposure to hydrocortisone, the character of both the cells and their secretion changed. In the central area production of the densely staining material almost or completely ceased, and instead a more diffuse granular secretion was formed which with azan stained patchily in purple, blue, and yellow but brightly with alcian blue. The peripheral goblet cells (Plate 4, fig. 23) became grossly distended with mucin which in azan-stained sections contained many orange granules; the resulting secretion was yellow with azan but intensely blue with alcian blue. In the less-differentiated central cells the goblets diminished in size and their place in the cell was occupied by very basophil cytoplasm. Meanwhile the cells of the germinative layer had proliferated and begun to form an epidermoid type of epithelium beneath the secretory cells which in places were being sloughed.

The explants transplanted to normal medium produced only mucus during the first 2 days, and in azan-stained preparations there was no sign of the reddish-orange secretion. At 4 and 6 days, however, there was much less difference between the cultures with and without the hormone. The secretion of the skin grown in normal medium after pretreatment with the vitamin had the same multicoloured appearance when stained with azan as in those treated with hydrocortisone and the secretory cells underwent similar changes to those described above. The basal cells were beginning to regenerate a squamous
epithelium beneath the secretory tissue, but the change was less advanced than in the explants grown in the presence of hydrocortisone.

**Feathers**

When the living cultures were examined 1 day after transfer from the high vitamin A medium, the feathers of those exposed to hydrocortisone were found to have behaved like the feather germs of cultures grown in the presence of both agents simultaneously. They had swelled into thin-walled balloons which continued to enlarge until the end of the 2nd day, after which they underwent little further change. In the explants grown in the absence of the hormone, however, there was no distension of the feathers which thus presented a striking contrast to those of the other series.

In sections of 2 explants fixed 2 days after transfer to the presence of hydrocortisone (Plate 2, fig. 8) the histological structure of the feathers was almost indistinguishable from that in skin grown in the presence of both agents simultaneously. The distended feathers were covered by and embedded in the dense secretion mentioned above. After 4 days they were well keratinized, and the solid neck by which they were attached to the skin had differentiated into a thick sheath enclosing folded barb primordia. By 6 days the balloons were crumpled and atrophic and the feathers had undergone no further development.

In the vitamin-A-treated explants transferred to normal medium and fixed after 2 days (Plate 2, fig. 9) the epithelium of the feather germs was secreting a little mucus, but by 6 days the tips of the rudiments had begun to keratinize. At this stage the sheath had differentiated and the infolding of epithelium to form the barbs had begun.

_The effect of transferring hydrocortisone-treated explants to medium containing added vitamin A_

Eight explants (Table 2, exp 341) were grown for 2 days in medium to which hydrocortisone had been added, and were then transferred to medium containing 10 i.u. vitamin A/ml. Sister explants were transferred from hydrocortisone to normal medium, others were cultivated first in normal medium and then exposed to vitamin A, and a 4th group were grown in normal medium throughout the experiment.

**Skin**

The explants pretreated with hydrocortisone for 2 days and grown for a further 2 days in the presence of vitamin A, resembled skin exposed continuously to hydrocortisone. The peridermal cells were greatly distended but contained many of the cytoplasmic granules stained reddish-orange with azan, that are a feature of the normal periderm. The epidermis had begun to keratinize, but keratinization did not progress, and by the 4th day the thin stratum corneum was being sloughed along with the periderm and some of the underlying cells.
Sections stained with alcian blue showed many scattered mucous cells particularly near the margin of the tissue, but there was no sign of the curious secretion produced in the explants treated first with vitamin A and then with hydrocortisone, or with both agents simultaneously.

In skin grown for 2 days in normal medium and then for 2 days in medium with vitamin A the epidermis was usually thinner and less differentiated than in the preceding series. The thin two-layered periderm sometimes had cilia-like processes near the periphery of the explant and elsewhere was being sloughed; the cells were not dilated. The true epidermis consisted of a basal columnar layer and one or two upper layers. After 4 days the epidermis was thicker and contained some mucin, though less than in the cultures pretreated with hydrocortisone.

**Table 3**

**Summary of effects of different treatments on the skin**

For experimental details see Tables 1 (groups 1-4) and 2 (groups 5-9). C, normal medium; HC, hydrocortisone; A, vitamin A; HC+A, both agents added together; → transfer from one type of medium to another; sq, squamous but not yet keratinized. In series A → C and A → HC, 4 of the results are marked with a ? because it was uncertain whether the superficial cells that produced the early secretion were of peridermal or epidermal origin (the former is more probable).

<table>
<thead>
<tr>
<th>Group</th>
<th>Periderm</th>
<th>Epidermis</th>
<th>Feathers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mucous secretion</td>
<td>Viscid secretion</td>
<td>Cilia-like processes</td>
</tr>
<tr>
<td>1. C</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. HC</td>
<td>+</td>
<td>+ (1 explant)</td>
<td>-</td>
</tr>
<tr>
<td>3. A</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4. HC+A</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>5. A → C</td>
<td>?++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6. A → HC</td>
<td>?++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7. C → A</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8. HC → C</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9. HC → A</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

The explants transferred from hydrocortisone-containing to normal medium rapidly differentiated into a typical squamous keratinized epidermis beneath the dilated peridermal cells which even after 2 days in normal medium were very degenerate and being shed.

The controls kept in normal medium throughout the experiment differentiated much more slowly than those pretreated with the hormone; the peridermal cells remained flat and compact, though they had begun to degenerate by the 4th day.

**Feathers**

The feather germs of the hydrocortisone-treated cultures changed little during the 4 days' cultivation in the presence of vitamin A. Although the ends of the larger rudiments were slightly more dilated than those of sister explants transferred to normal medium, they did not show the remarkable ballooning seen in cultures grown in the presence first of vitamin A and then of hydrocortisone, or of both agents together. The feathers may have begun to keratinize at the time of transfer to medium containing vitamin A; 2 days after transfer they were well
keratinized and the stratum corneum had increased in thickness by the 4th day. That keratinization was not arrested in the feather germs as it was in the skin, was probably due to poor penetration of the vitamin into the elongated feather with its impermeable horny surface.

The feathers of explants grown first in normal and then in vitamin-A-containing medium continued to develop slowly but did not keratinize.

In the controls kept in normal medium throughout the experiment, the feathers developed as described in section 2. Their keratinization was much less advanced than in corresponding explants pretreated with hydrocortisone before transplantation to normal medium.

DISCUSSION

The periderm is very versatile in its response to different environmental conditions (Table 3). In the normal embryo it has at first a secretory function and the upper layer produces a mucous substance. Later, irregular cytoplasmic granules with the staining reactions of keratin appear, mainly in the lower layer, and eventually fill the cells, while the upper stratum becomes very attenuated (Fitton Jackson, personal communication). Near the end of embryonic life the periderm degenerates and is sloughed from the definitive epidermis which differentiates beneath it.

In control explants in normal medium the periderm continues at first to secrete alcian-blue staining material, presumably of mucoid character, develops its characteristic cytoplasmic granules, and eventually degenerates and is shed from the stratum corneum of the epidermis in the normal way. Hydrocortisone alone does not inhibit the secretory activity of the periderm, but causes the cytoplasmic granules to appear precociously in the lower layer; the gross dilation of the cells during the first 2 days suggests that their permeability also may have been affected. Under the influence of vitamin A alone both the secretory and mitotic activities of the periderm are much prolonged and, especially with the lower dose of vitamin (5 i.u./ml.), many of the superficial cells formed processes resembling cilia; whether these processes were true cilia or very large microvilli could not be determined. The periderm does not become sharply demarcated from the epidermis as in normal development, but forms an integral part of the epithelium.

The most striking effect on the periderm is produced by the combined action of the two agents. Hydrocortisone alone sometimes causes the secretion of a peculiar refractile, viscid substance which reacts intensely with PAS and stains like keratin with azan. When the vitamin also is present, this secretion occurs in nearly all the explants and is often very copious. The chemical composition of the substance is unknown. That it contains material related to keratin is suggested both by its staining reaction with azan and by the fact that autoradiographs have shown it to be rich in cystine (Pelc & Fell, unpublished). It probably contains acid mucopolysaccharide also, since some inorganic sulphate is present.
(Pelc & Fell, unpublished); the material also stains, though weakly, with alcian blue and strongly with PAS after digestion with diastase.

As stated above, the periderm in the normal embryo forms irregular cytoplasmic granules which stain like keratin with azan and increase in amount as differentiation proceeds; Matoltsy (1958) has shown that the older periderm is rich in SH-containing protein. Hydrocortisone alone accelerates the appearance of the peridermal granules in the explants, and it seems probable that the viscid secretion formed in the presence of the hormone and the vitamin together, may be related to these organelles; against this view is the fact that whereas the secretion is intensely PAS-positive, the normal peridermal granules are nearly colourless with this stain (Fitton Jackson, unpublished).

The epidermis also shows a wide range of histological reactions. In normal medium it differentiates more rapidly than in the embryo (cf. Miszurski, 1937; Fell & Mellanby, 1953), but in the presence of hydrocortisone development is still more precocious; the hormone causes a similar acceleration of differentiation in explants of foetal rat skin (Weissmann & Fell, 1962). On the other hand, vitamin A, even at the low level of 5 i.u./ml., completely inhibits keratinization and promotes the secretion of mucus (cf. Fell & Mellanby, 1953; Fell, 1957; Fell & Pelc, 1960).

As in the experiments on limb-bone rudiments in culture previously reported by Fell & Thomas (1961), vitamin A and hydrocortisone added together to the medium, are mutually antagonistic in their effect upon the epidermis. This results in a curious struggle for supremacy between the two compounds, which is particularly striking when the lower dose of vitamin (5 i.u./ml.) is used. Under these conditions the hormone may at first prevail, so that a squamous keratinizing epithelium may differentiate over nearly the whole surface of the explant. Sometimes a keratinizing area may be continuous with one that is producing the same type of viscid secretion as was formed by the periderm at an earlier stage, or keratinizing epidermis may be contiguous to non-keratinized mucous epithelium. Eventually, as in the limb-bone rudiments, the vitamin wins the battle, probably because it accumulates in the cells, and the keratin and viscid material formed previously, are sloughed. The histological picture suggested that when the conflict between the two agents is evenly balanced, the effect on the cells is more harmful than when the vitamin readily prevails over the hormone causing a fairly rapid mucous metaplasia.

When explants grown for 8 days in the presence of vitamin A are transferred to medium to which hydrocortisone has been added, we again see the profuse secretion of viscid keratin-like material during the first 2 days after transfer; it is not clear whether this substance is formed by persistent periderm or by superficial epidermal cells, but the former alternative is the more likely. The viscid secretion does not appear in sister explants transplanted from hyper-vitaminotic to normal medium. This first secretory phase is followed by a second in which apparently similar material is produced by both the skin exposed to
hydrocortisone and that placed in normal medium. During this second phase, in both series mucous secretion at first became much more profuse, and fully developed goblet cells differentiated (cf. Fell & Mellanby, 1953; Fell, 1957); the alcian blue staining material, probably an acid mucopolysaccharide, however, becomes mixed with an increasingly large proportion of a substance that has the same staining reactions as the secretion produced during the first 2 days in the presence of hydrocortisone. This suggests the possibility that as the vitamin A disappears from the cells in both the normal and hydrocortisone-containing medium, they begin to synthesize materials related to keratin rather than acid mucopolysaccharide. Eventually in both series the secretory cells are shed and replaced by a squamous epithelium regenerated by the basal cells.

Explants grown in the presence of hydrocortisone and transferred to A-hypervitaminotic medium do not form the early viscid secretion. This is probably due to the fact that under the influence of the hormone, the periderm has begun to regress before the tissue is exposed to the vitamin, and the superficial cells of the epidermis proper are too highly differentiated to respond in this way.

The behaviour of the feather germs (Table 3) varies greatly under the different experimental conditions used. In normal medium, sheath and barb components develop, a follicle is formed, and the sheath keratinizes, but differentiation does not progress beyond this point; growth is inhibited probably owing to poor nutrition in the absence of a vascular system, since little food would pass through the horny sheath.

Hydrocortisone greatly accelerates the histological differentiation of the feathers as compared with those in the control explants, and arrests their elongation almost immediately. This result confirms the observations of Karnofsky, Ridgway, & Patterson (1951) and Moscona & Karnofsky (1960) who found that feather formation was inhibited in chick embryos treated in ovo with cortisone. Moscona & Karnofsky thought that this was probably due to a direct action of the hormone on the tissue, a view that the present results have shown to be correct. Under the influence of vitamin A the feathers enlarge but undergo little further differentiation and fail to keratinize.

When exposed to the hormone and the vitamin simultaneously, the larger feather germs quickly swell to several times their original size; the same effect is produced when skin grown for 8 days in the presence of vitamin A is transplanted to medium to which hydrocortisone alone had been added. The hormone must cause a rapid change in the permeability of the unkeratinized feathers to produce this striking effect which did not appear either in sister explants transplanted from hypervitaminotic to control medium, or in the keratinizing feathers of skin grown for 2 days in the presence of hydrocortisone and then exposed to vitamin A.

The physiological mechanism whereby hydrocortisone antagonizes the effect of vitamin A on the epidermis is not known. Recent work by Lucy, Dingle, & Fell (1961), Dingle, Lucy, & Fell (1961), and Dingle (1961) indicates that
vitamin A causes the degradation of cartilage matrix in culture by liberating proteases from the lysosomes of the cells. Fell & Thomas (1960) found that hydrocortisone much delays the action of the vitamin on cartilage in culture, and Weissmann & Dingle (unpublished) have shown that the release of proteases by vitamin A from the large granule fraction of rat liver is retarded by pretreatment of the fraction with hydrocortisone. It is possible, therefore, that the hormone may inhibit the action of the vitamin on cartilage, by preventing the liberation of hydrolases from the lysosomes.

As a result of autoradiographic studies with radio-active cystine and inorganic sulphate, Pelc & Fell (1960) suggested that 'basal cells and early differentiated cells in keratinizing epithelium have a mechanism for producing sulphated mucopolysaccharides; this mechanism is blocked by keratinization, but not when keratinization is stopped by excess vitamin A'. It is conceivable that under the influence of vitamin A, lysosomal proteases are released which break down the precursors of keratin in the epidermal cells, thus inhibiting keratinization and permitting the continued synthesis of sulphated mucopolysaccharides; the release of these hydrolases may be inhibited by hydrocortisone. It must be emphasized that this suggestion is mere speculation, but the question is now being investigated.

SUMMARY

1. The effects of hydrocortisone, vitamin A, and both agents together were compared in explants of skin from 9-day chick embryos grown in organ culture.
2. In normal medium the periderm degenerated and was shed in the usual way. The epidermis differentiated and keratinized more rapidly than in the embryo; the feather germs differentiated into sheath and barb rudiments and keratinized, after which development ceased.
3. Hydrocortisone alone caused extreme dilation of the peridermal cells which later were sloughed. The differentiation of the epidermis was much accelerated as compared with that of controls in normal medium. The feather germs aborted at an early stage, probably owing to precocious histological differentiation.
4. With vitamin A alone the peridermal cells actively secreted mucus and many formed cilia-like processes; the periderm formed an integral part of the epithelium. In the epidermis keratinization was suppressed and mucous cells differentiated. The feathers remained almost unchanged; the epithelium failed to keratinize and often became secretory.
5. In explants exposed to both agents together, during the first 2 days the periderm secreted copiously a dense, viscid substance the nature of which was unknown, but which appeared to contain both keratinous and mucous material; a similar secretion was sometimes produced by the epidermis at a later stage when the concentration of added vitamin A was low (5 i.u./ml.). The hormone and the vitamin were mutually antagonistic in their action on the epidermis. At first the hydrocortisone prevailed, and large areas of the epithelium often became
squamous and keratinized; later the vitamin predominated, the keratin was sloughed, and mucous cells were formed.

6. In two days, feather germs exposed to both agents at once, swelled to several times their original size.

7. Changes like those produced by the hormone and vitamin together appeared in explants grown for 8 days in the presence of vitamin A and then transferred to medium containing hydrocortisone, but not in skin pretreated with the vitamin and then placed in normal medium.

8. These effects were not seen in skin transplanted from medium with hydrocortisone to one containing vitamin A; the epidermis showed the usual changes produced by vitamin A alone.

**RÉSUMÉ**

*Influence de l'hydrocortisone sur l'action métaplastique exercée par la vitamine A sur l'épiderme de la peau d'embryon de Poulet en culture d'organes*

1. Les effets de l'hydrocortisone, de la vitamine A et de ces deux agents associés ont été comparés sur des explants de peau d'embryons de Poulet de 9 jours en culture d'organes.

2. Dans un milieu normal, le péridermé dégénère et s'élimine comme d'habitude. L'épiderme se différencie et se kéranitise plus vite que dans l'embryon; les germes plumaires se différencient en une gaine et en ébauches de barbes, elles se kéranitisent, puis leur développement cesse.

3. L'hydrocortisone seule provoque une très forte dilatation des cellules péridermiques qui ensuite se desquament. La différenciation de l'épiderme est beaucoup accélérée par rapport aux témoins en milieu normal. Les germes plumaires avortent à un stade précoce, ce qui est probablement dû à une différenciation histologique prématurée.

4. En présence de vitamine A seule, les cellules péridermiques sécrètent activement de la mucine, beaucoup d'entre elles deviennent ciliées; le péridermé fait partie intégrante de l'épithélium. La kéranisation de l'épiderme est supprimée et des cellules muqueuses se différencient. Les plumes ne subissent presque aucune modification; l'épithélium ne se kéranitise pas et devient souvent sécrétoire.

5. Dans des explants exposés aux deux agents à la fois, le péridermé sécrète abondamment, pendant les deux premiers jours, une substance visqueuse de nature inconnue, mais qui paraît contenir du matériau à la fois kéranitieux et muqueux; une sécrétion analogue est parfois élaborée par l'épiderme à un stade ultérieur quand la concentration de la vitamine A est faible (5 u.i./ml.). L'hormone et la vitamine sont antagonistes l'une de l'autre en ce qui concerne leur action sur l'épiderme. C'est d'abord l'hydrocortisone qui prédomine, et de larges aires de l'épithélium deviennent souvent squameuses et se kéranisent; plus tard la vitamine A prédomine, la kéranine est éliminée et des cellules muqueuses se forment.
6. En deux jours, les germes plumaires exposés en même temps aux deux agents se gonflent et atteignent plusieurs fois leur taille originelle.

7. Des transformations comme celles que produit l'apport simultané d'hormone et de vitamine apparaissent dans des explants cultivés 8 jours en présence de vitamine A, puis transférés dans un milieu contenant l'hydrocortisone, mais non dans une peau traitée au préalable par la vitamine et placée ensuite en milieu normal.

8. On n'observe pas de tels effets sur une peau transplantée d'un milieu contenant de l'hydrocortisone dans un milieu contenant la vitamine A; l'épiderme montre les transformations usuelles provoquées par la vitamine A seule.

ACKNOWLEDGEMENTS

The author is indebted to Mr. L. J. King for his valuable technical assistance, to Dr. S. Fitton Jackson for much helpful discussion, and to Dr. Lewis Thomas who provided the hydrocortisone.

REFERENCES


EXPLANATION OF PLATES

(Photographs by Mr. M. Applin, except fig. 20 by Dr. S. Fitton Jackson)

PLATE 1

FIG. 1. Feather germs of normal 9-day embryo. Azan. x60.

FIG. 2. Feather germs of control explant grown for 2 days in normal medium (exp. 334); follicles (f) have begun to develop. Azan. x60.

FIG. 3. Feather germs of skin grown for 2 days in medium containing hydrocortisone (7.5 µg./ml.) (exp. 334). The feathers are abortive; note their precocious histological differentiation into sheath (s) and barb (b) rudiments and the deep follicles (f). The peridermal cells (p) are greatly swollen. Azan. x60.

FIG. 4. Feather germs of skin grown for 2 days in the presence of vitamin A (10 i.u./ml.) (exp. 334). The feather germs have enlarged slightly but undergone no further development; in life they are flabby and tend to rest on the surface of the epidermis (see middle rudiment). The ‘ghost’ of the rayon fabric (rf) which has been dissolved from the tissue, can be seen. Azan. x60.

FIG. 5. Feather germs grown for 2 days in medium containing both hydrocortisone and vitamin A (10 i.u./ml.) (exp. 334). Note the enormous dilation of the feathers. A little viscid secretion (s) is present between the feathers and the skin. Azan. x60.

FIG. 6. Similar explant exposed for 2 days to hydrocortisone and the lower dose of vitamin A (5 i.u./ml.) (exp. 335), showing a thick layer of viscid secretion (s) produced by the periderm. The small feather papillae are not dilated like the larger rudiments in fig. 5. Azan. x100.

FIG. 7. Skin exposed to both hydrocortisone and the lower concentration of vitamin A (5 i.u./ml.) for 4 days (exp. 310). The epidermis (ep) itself has secreted a thick layer of the viscid material (s) seen in fig. 6. Azan. x60.

PLATE 2

FIG. 8. Explant grown for 8 days in the presence of vitamin A (10 i.u./ml.) and then transferred for 2 days to medium containing hydrocortisone (exp. 307). Much viscid secretion (s), like that in figs. 6 and 7, has been produced and the feather-germs are greatly dilated. Azan. x60.

FIG. 9. Sister explant to that shown in fig. 8, grown for 8 days in medium with added vitamin A and then transplanted to normal medium for 2 days (exp. 307). No viscid secretion has been formed and the feather germs are not dilated; note their horizontal position. Azan. x60.

FIG. 10. Epidermis of normal 9-day embryo, showing two-layered periderm (p) resting on columnar epidermal cells. Azan. x510.

FIG. 11. Epidermis of control explant grown for 8 days in normal medium (exp. 305). The epidermis has formed a squamous epithelium with a thin stratum corneum (sc) above which are the remains of the periderm (p). Azan. x510.

FIG. 12. Epidermis of sister explant grown for 8 days in the presence of hydrocortisone (exp. 305). The stratum corneum (sc) is much thicker than that in fig. 11; most of the periderm (p) has been sloughed. Azan. x510.

FIG. 13. Epidermis of sister explant grown for 8 days in the presence of vitamin A (10 i.u./ml.) (exp. 305). No keratin has been formed, and the superficial layer consists of cubical secretory cells, one of which is in mitosis (mi). Azan. x510.

FIG. 14. Another section of the same explant as that in fig. 14, stained to show early mucous secretion (m). Mayer’s acid haemalum, alcian blue. x510.

PLATE 3

FIG. 15. Epidermis of explant grown for 6 days in medium with the lower concentration of vitamin A (5 i.u./ml.) (exp. 335). Note peridermal cells with cilia-like processes (c). Azan. x510.

FIG. 16. Epidermis of explant grown for 10 days in the presence of the lower concentration of vitamin A (5 i.u./ml.) (exp. 335). Note the well-developed secretory layer of columnar cells. One of the basal cells is in mitosis (mi). Azan. x510.

FIG. 17. Another section of the same explant stained to show mucus (m) in the young goblet cells. Mayer’s acid haemalum, alcian blue. x510.

FIG. 18. Epidermis of explant cultivated for 2 days in medium containing both hydrocortisone and vitamin A (10 i.u./ml.) (exp. 334). The periderm (p) is actively secreting viscid material (s). Azan. x510.

FIG. 19. Epidermis of skin cultivated for 4 days in medium to which both hydrocortisone and vitamin A in the lower concentration (5 i.u./ml.) had been added (exp. 310). The epidermis itself is secreting the viscid material (s) which is intensely PAS-positive. Note the PAS-staining material (cs) in the epidermal cells. PAS, after digestion with diastase, and Mayer’s acid haemalum. x1,300.
H. B. FELL

Plate 2
FIG. 20. Epidermis of explant grown for 6 days in medium containing both hydrocortisone and vitamin A in the lower concentration (5 i.u./ml.) (exp. 335). In this region the hormone has prevailed over the vitamin and a squamous well keratinized (sc) epithelium has differentiated. Azan. × 510.

FIG. 21. The same section as in fig. 20 near the margin of the explant; here the vitamin has prevailed and there is no keratinization. × 510.

FIG. 22. Epidermis of explant grown for 10 days in medium with hydrocortisone and the lower level of vitamin A (5 i.u./ml.) (exp. 335). Keratin formed at an earlier stage in response to the hormone (cf. fig. 20), has now been sloughed under the growing influence of the vitamin. Azan. × 200.

FIG. 23. Explant grown for 8 days in medium containing vitamin A (10 i.u./ml.) and then transplanted for 6 days to medium with hydrocortisone (exp. 307). The superficial cells near the periphery of the explant are copiously secreting mucus (m) instead of the viscid substance found at 2 days (cf. fig. 8). The basal cells have begun to form a squamous epithelium (sg) beneath the goblet cells. Mayer's acid haemalum, alcian blue. × 510.

(Manuscript received 14: xii: 61)