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METAPLASIA PRODUCED IN CULTURES OF CHICK ECTODERM BY HIGH VITAMIN A

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One of the earliest discoveries concerning vitamin A was that a deficiency of this substance affected the epithelium of the eye and caused keratomalacia and xerophthalmia. Mori (1922*a*, *b*, 1923) extended this observation by examining the paraocular glands and other tissues of A-deficient rats and found that the epithelium of the ducts of these glands and of the larynx and trachea underwent metaplastic changes and became keratinizing stratified epithelium. Wolbach & Howe (1925) confirmed the results of Mori in rats, and in guinea-pigs (1928), and found that in vitamin A deficiency, stratified keratinizing epithelium replaced normal epithelium in the respiratory tract, genito-urinary tract, salivary glands, the paraocular glands and the pancreatic ducts. In this work they gave a detailed account of the histo-pathology of the metaplastic changes. In 1933 they followed up this investigation by describing the reparative histological changes which accompany the replacement of the keratinizing epithelium by epithelium normal to the position when vitamin A is added to the vitamin-deficient diet of rats.

More directly related to the present work is the effect of abnormally high concentrations of vitamin A on skin epithelium as described recently by Studer & Frey (1949) and by Sabella, Bern & Kahn (1951). Studer & Frey gave massive doses (40,000 i.u.) daily by mouth to rats, and produced a thickening of the epidermis especially of the stratum granulosum. The normally flattened cells of the stratum germinativum also proliferated and became cubical, and other striking epidermal changes were noted. After about 12 days, the epithelium began to revert to its normal state and the reversion was complete in 21 days although the same treatment was continued. Sabella *et al.* applied vitamin A alcohol in sesame oil (in concentration 5000 i.u./ml.) directly to the skin of rats; 10 days of this treatment caused local thickening of the

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epidermis, particularly of the stratum granulosum, and a decreased rate of keratin-formation with an increased production of kerato-hyalin. They remark that both their own experiments and those of Studer & Frey suggest, but do not establish, the ability of vitamin A in high concentrations to counteract keratin-formation in the skin epidermis. It is evident from these experiments that increased concentrations of vitamin A, whether systemically given or locally applied, have a definite effect on skin epidermis.

The ectoderm of the embryonic chick is known to develop normally though precociously in tissue culture (Miszurski, 1937) and to produce large quantities of keratin. The following experiments were undertaken to see what effect the addition of excess vitamin A to the medium would have on keratinization in such cultures. The results to be described showed that cornification was completely suppressed by the high vitamin A, but a quite unexpected effect was also produced. In the high A medium, the ectoderm differentiated into a mucus-secreting sometimes ciliated membrane resembling that of the normal nasal mucosa, and when the explants were transferred to normal medium, this metaplastic change was reversed, and the natural keratinizing epithelium of the skin was produced.

Two main groups of experiments were made. In the first (described in section I) the effect of excess vitamin A on the differentiation of the ectoderm was studied; the explants derived from one side of each embryo were grown in medium to which vitamin A had been added (+A medium) and those from the opposite side in normal (control) medium. The second group of experiments (see section II) were undertaken to find whether the effect of the vitamin was reversible; the explants from both sides of each chick were grown in +A medium for periods ranging from 7 to 14 days; the explants from one side were then transferred to normal medium and those from the other were either fixed to show the histology of the tissue at the time of transfer to normal medium, or were cultivated in +A medium until the sister cultures in normal medium were fixed.

MATERIAL AND METHODS

Material

The explants were obtained from the trunk and limbs of 6- to 7-day chick embryos. The head, viscera and distal part of the limbs were removed; the body was then laid with the epidermal surface upwards and the spinal region with its associated ectoderm and the thin ventral body wall were cut away and rejected. Each half of the trunk and the attached portions of the limbs were cut into large fragments from which as much as possible of the skeletal tissue was dissected; the surface fragments were then cut into smaller pieces suitable for explantation. The explants from one half embryo were kept separate from those of the other half.

Vitamin A

In all the experiments in the present work, the vitamin A was added to the plasma either in the form of the alcohol or acetate, to produce what in our previous work we have described as artificial hypervitaminotic (AH) plasma. The vitamin A alcohol solution was prepared from a sample of

avoleum (kindly supplied by British Drug Houses Ltd.) containing 1,500,000 i.u./g. This was dissolved in ethanol to give a solution containing 20,000 i.u./ml. The ethanol solution was then added to normal plasma to raise the vitamin A content to the desired amount, which varied in different experiments between 1000 and 3000 i.u./100 ml. In adding the extra vitamin A, the amount present in the plasma itself must be taken into account, and this for normal fowls varied between 200 and 350 i.u./100 ml. When vitamin A acetate was added, the crystalline substance was dissolved in ethanol to give concentrations similar to those obtained with vitamin A alcohol. The strength of the solution, both of the vitamin A alcohol and the vitamin A acetate, was such that the plasma finally contained about 0·2% ethanol; the same quantity of ethanol was added to the normal control plasma.

Because of the instability of the vitamin A in alcoholic solution under some conditions, it was necessary, after adding the vitamin A alcohol or acetate to the plasma in the calculated amount, to determine its vitamin A content again. This was especially important with vitamin A acetate in ethanol, since a stock solution, even when kept in the dark at 0° C, soon loses its potency. Vitamin A alcohol is fairly stable in plasma, but the acetate slowly diminishes so that, for instance, AH plasma containing 2040 i.u. vitamin A per 100 ml. may, at the end of 7 days, contain only 1459 i.u. The estimation of the vitamin in plasma was made colorimetrically (Fell & Mellanby, 1952).

It may be pointed out that the vitamin A, whether added as alcohol or acetate, is not in the identical physical state normally present in plasma. In normal plasma, the vitamin A is in a water-soluble form probably in combination with a protein as a lipoprotein, and can only be removed from the plasma by petrol ether extraction as a fat-soluble substance after the plasma proteins have been denatured by 50% ethanol. On the other hand, vitamin A alcohol and acetate when added in alcoholic solution to plasma can be extracted directly by shaking the plasma with petrol ether. Thus in the enriched plasma used in the present experiments, the greater part of the natural vitamin A was water-soluble (250–350 i.u./100 ml.), whereas the remainder was fat soluble (2250–2150 i.u./100 ml., on the assumption that the total plasma content was 2500 i.u./100 ml.).

In our previous work on bone development in tissue culture (Fell & Mellanby, 1952), it was found that vitamin A in artificial hypervitaminotic plasma had the same kind of action as a similar concentration of vitamin A in natural hypervitaminotic plasma, but that the former was much more effective and influenced the bone changes more rapidly. In other words, this action of vitamin A was greater in the fat-soluble form than in its natural water-soluble combination.

Tissue culture

The culture medium consisted of three parts of fowl plasma: one part of a concentrated extract of a 13- to 14-day chick embryo; the Tyrode solution with which the extract was made contained 1% glucose, giving a concentration of 0·25% in the final medium. The best results were obtained with the vitamin A acetate; although the vitamin A alcohol produced the same changes in the ectoderm as the acetate, it seemed to be slightly toxic and caused rather more degeneration from the 9th day onwards.

Watch-glass cultures (Fell & Robison, 1929; Fell & Mellanby, 1952) and large hanging-drop preparations were used. In the former, nine or twelve drops of plasma were mixed with three or four drops of embryo extract. The tissue fragments were first placed in embryo extract made with Tyrode containing the usual concentration (0·1%) of glucose and diluted to half the strength of the extract in the culture medium; they were then explanted, with the ectoderm uppermost, on the surface of the clot in the watch-glass and the excess extract was sucked off with a fine pipette. The tissue was removed from the old clot and transplanted to fresh medium four times a week; it was found desirable to cut off the sheets of epithelium which spread over the clot since during transplantation they sometimes became folded over the under surface of the explant, keratinized and obstructed nutrition.

The hanging-drop preparations were made with $1\frac{1}{4}$ in. square cover-slips and $3 \times 1\frac{1}{2}$ in. hollow ground slides. The explants were grown in a mixture of 0·06 ml. of plasma and 0·02 ml. of embryo extract per cover-slip and were subcultivated 3 times a week.

Number of cultures studied. In the first main group of experiments (section I), thirty-eight +A explants and twenty-eight controls grown in watch-glasses and nineteen +A and twenty-three controls from hanging-drop cultures were histologically examined after periods of cultivation ranging from 7 to 14 days. In the second group, eight explants from watch-glasses and fourteen from hanging drops were fixed and sectioned after being grown in the +A medium for 7–14 days and then transferred to normal medium for 4–8 days.

Histology

The explants were fixed in 3% acetic Zenker's fluid; fixation for 30 min gave the best results. For comparison with the explants, the ectoderm, with underlying body wall, and the nasal regions of 7-, 10-, 13- and 18-day embryos, and also the skin and nasal mucosa from an adult cock were fixed in 5% acetic Zenker's fluid for periods varying according to the size of the tissue fragment. After being washed, dehydrated and cleared in Cedar wood oil in the usual way, the tissue was embedded in paraffin wax and sectioned at a thickness of 8 μ.

Sections were stained by one of the following procedures: Bauer's and Bensley's modification of the Periodic acid–Schiff reaction (PAS), after digestion with saliva, followed by Meyer's haematoxylin or haemalum, or by mucicarmine followed by Meyer's haematoxylin, by Delafield's haematoxylin and chromotrop, or with azan and celestin blue, followed by haemalum and van Giesen's stain. The sections were mounted in Depex.

RESULTS

I. *The effect of cultivation in +A medium*

The structure of the original explants

In the original explants (Pl. 1, fig. 3) the ectoderm consisted of an outer layer of flattened cells and a basal layer of columnar or cubical cells which varied in depth in different parts of the trunk and limbs. Feather papillae had not yet appeared. Below the ectoderm was loose mesenchymatous tissue.

Watch-glass cultures

Controls. During the first 48 hr in normal medium the tissue spread into a sheet composed of mesoderm, with a marginal fringe of amoeboid cells, and a superficial layer of ectoderm which often extended beyond the mesoderm as a thin membrane on the surface of the clot. By about the 4th day small, dome-shaped feather papillae had appeared.

Between the 4th and 7th day the central region of the explants had greatly thickened and sometimes projected from the clot like a balloon, from the base of which epithelium and fibroblasts wandered over and into the medium. The epidermis increased in thickness, and by the 7th day was often much folded and, especially in the peripheral part of the tissue, had become opaque owing to cornification of the superficial layers. The feather germs had elongated and assumed a fairly symmetrical bottle-shape, the 'neck' being attached to the skin. In cultures of embryos from a pigmented breed, branched melanophores differentiated and wandered into the epidermis and especially into the feather germs, which often looked nearly black to the naked eye. By the 9th day the controls were almost completely epithelialized, only a narrow gap

remaining from which fibroblasts continued to wander and from the edge of which epithelial sheets spread over the coagulum. Keratinization rendered the epidermis very opaque. The controls were not maintained for longer periods.

Sections showed that the differentiation of the 6- to 7-day ectoderm in normal medium was precocious as compared with its development in the embryo (cf. Miszurski, 1937). Thus after 7 days *in vitro* (Pl. 1, fig. 1) the Malpighian layer was three to four cells deep over the central part of the explant and the superficial stratum had begun to cornify, while near the periphery the epidermis was even thicker and had often produced several completely keratinized layers. In the normal 13-day embryo, on the other hand, the ectoderm was still only two-layered and keratinization had not begun.

The feather germs, however, were stunted and retarded as compared with those of the 13-day chick *in ovo*, though they had undergone considerable development *in vitro* and were set in well-defined follicles. For most of their length they were composed of a thick, multi-layered epidermis, in which the rudiments of the barbs were sometimes recognizable as ridge-like thickenings on the inner surface, enclosing a pulp of reticular connective tissue; the tips of the feathers consisted of a solid mass of undifferentiated epithelium. In the pigmented cultures, branching melanophores abounded in the feathers.

Two regions were distinguishable in the connective tissue (Pl. 1, fig. 1): a broad outer zone of reticular tissue corresponding to the dermis, sharply demarcated from an inner, compact, rather fibrous mass derived from the subdermal tissue and sometimes containing nodules of cartilage and myoblasts. As already described, some of the control explants were almost completely epithelialized by the 7th day, forming a 'balloon' from the narrow mouth of which cells migrated into the clot. In such cultures the epidermis was wholly or partly encased by a keratinized shell, and was usually atrophic or degenerate, while most of the inner zone of connective tissue was necrotic. Of the 9-day controls, three were completely encapsuled and very degenerate but the remaining three, though well keratinized and at the 'balloon' stage, were fairly healthy.

+A explants. During the first 4 days the +A cultures behaved like the controls in normal medium, but the epidermis failed to keratinize. By the 7th day it was still translucent and fairly thin; the sub-epidermal tissue had acquired a bloated oedematous appearance, and the feather germs, though much enlarged, were distorted, swollen and often fused laterally with each other. Like the controls, the +A explants had become almost completely epithelialized by the 9th day and in some cultures the epidermis had lost its smooth contour in places and acquired a curious 'fluffy' appearance.

After 11–14 days in the +A medium, the oedematous condition of the explants seen earlier in cultivation had disappeared, and the connective tissue

had become very compact and dense. The 'fluffy' appearance of the epidermis was now very pronounced in some cultures and in others, though still regular, the epithelium was much thickened; there was still no sign of keratinization. A strange feature of this stage was the presence of a translucent, viscid envelope round the explants, which clung to the tissue during transplantation and could not be completely washed away with saline. This material was not composed of cell debris, although it contained some cellular remains, and until the explants were examined histologically its nature remained obscure.

Histological examination of the +A cultures showed that keratinization was completely suppressed. The epidermis of the 7-day explants was three to four cells deep (Pl. 1, fig. 4). In places the superficial layer was flattened as in the controls, but elsewhere, especially near the periphery of the culture, the cells were cubical and the surface of the cytoplasm was coloured a deep violet with Delafield's haematoxylin and chromotrop, and bright red with PAS (Periodic acid-Schiff reaction) or mucicarmine, so that in section the epithelium was bordered by a sharply defined purple or red line. This appearance was never seen in similarly stained controls of the same age. In the thickened epithelium near the periphery of the explant, scattered vacuolated cells and small round cavities were present which were filled with material having the same staining reactions as the epithelial border. Mitosis was common in all layers of the epidermis.

The tissue below the epithelium (Pl. 1, fig. 2) had the same general structure as in the controls, i.e. it consisted of an outer dermal and an inner, compact fibrous zone; in the +A explants, however, the outer zone was much more oedematous, and in places the epidermis was sometimes detached from the dermis to form a blister filled with fluid.

After 9 days in +A medium, the epithelium bore little resemblance to the keratinizing, squamous epidermis of the controls, but had assumed the characteristics of a secretory membrane. It had become thicker and the superficial flattened layer had disappeared and been replaced by cubical or, especially near the periphery of the explant, deep columnar cells. In azan-stained preparations many small red granules were seen immediately within the free border of these cells, and below the granules were large vacuoles which stained blue with azan and bright red with PAS or mucicarmine.

Many of the secretory cells protruded from the surface of the epithelium, and in places had escaped from their orderly arrangement to form irregular cords and clusters loosely adhering to the palisade of columnar cells representing the original basal layer; it was this disorderly structure which caused the 'fluffy' appearance described in some of the living explants. The loss of cohesion between the epithelial cells was not associated with necrosis, and the papilliform masses thus produced were secreting actively and appeared healthy. This change in structure probably increased the permeability of the

epithelium and may have been responsible for the reduction of the oedema in the connective tissue, which by the 9th day had become more compact and fibrous. Small round intercellular vacuoles full of secretion were common throughout the epithelium which here and there had invaginated into the 'dermis' to form structures resembling simple glands, the cells of which were pouring secretion into the lumen.

In explants where epithelialization was nearly complete, the cells were secreting less actively and contained no mitoses, while the enclosed connective tissue was largely necrotic.

The epithelium of the 14-day cultures showed the changes described above to an even greater degree, and in addition tracts of cells (Pl. 1, fig. 5) were seen wholly or partly detached from the basal cells and sometimes ciliated. In places the loose cells had been completely shed and the basal layer transformed into columnar secretory epithelium (Pl. 3, fig. 13). The secretory vacuoles in the cytoplasm were much larger than those in the 9-day explants and had the same staining reactions, as also had secretory material clinging to the free surface of the tissue.

Hanging-drop cultures

Controls. As in the watch-glass cultures, during the first 2 days in normal medium the control explants spread into a thin layer with a marginal zone of migrating fibroblasts, but although some epithelial sheets were formed, most of the ectoderm began to fold back on itself to produce large, irregular islets and cords surrounded by connective tissue. By the 4th day, fibroblastic outgrowth was profuse, fewer epithelial membranes were formed and in both series of cultures many of the epithelial islets had acquired a lumen into which feather germs often projected.

The innermost cells of the ectodermal masses, which corresponded to the superficial layers of the normal epidermis, were seen to be flat and opaque in optical section and were obviously beginning to keratinize. By the 7th day the ectoderm had developed into abnormally thick, squamous epithelium which was actively shedding concentric layers of keratin into the cavity of the cyst. After 9–14 days this cornified material had become so plentiful and dense that it appeared to the naked eye as white spots in the tissue.

Since the ectoderm usually folded back on itself to form cysts with the Malpighian layer outwards, the epidermis of the controls did not become invested by keratinized material as in the watch-glasses, and on histological examination it was found to remain healthy and active for longer periods. There was usually some degeneration in the deepest parts of the explant next to the glass, but until about the 14th day this was not extensive.

As in the watch-glass cultures, the development of the ectoderm in normal medium was precocious by comparison with the skin of the embryonic chick

in ovo, and keratinization much more extensive. The youngest control fixed had been growing for 9 days. This explant was one of the few in which the ectoderm had not formed a closed vesicle, but remained as a continuous sheet over the connective tissue. It had undergone a typical squamous differentiation; in the central region of the explant it was four to five cells deep, had begun to keratinize and cornifying feather germs projected from its surface. At the margin it was much thicker and had produced many layers of keratin. Most of the controls were fixed after 11 days (Pl. 3, fig. 15). The majority were cystic; in places the epithelial lining of the vesicles was seven to eight layers in depth, and in that part of the islet which was nearest to the margin of the culture where nutritional conditions were best, it had produced a dense stratum corneum which nearly filled the irregular, rather flattened cavity. Nearer the centre there was often little or no keratinization and in the deepest part of the culture where the epithelium was close to the coverglass, it was frequently rather necrotic. Feather germs covered by cornifying epithelium often projected into the cavity of the islets; some were degenerate, probably owing to their being largely isolated from a food supply.

The epidermis was surrounded by loose connective tissue which was condensed into fibrous capsules around the islets and in which ran many vascular channels lined by endothelium.

In a few of the 9-day and many of the 14-day controls, the epithelium was almost completely cornified so that the whole islet was converted into a 'pearl' of keratin, sometimes with a thin outer layer of atrophic or necrotic epithelium. In some of the 14-day explants, however, the epidermis though heavily keratinized, appeared healthy and active and mitosis was still present in the basal cells.

+A explants. In +A medium also, most of the ectoderm folded upon itself to form islets like those of the controls, but these islets developed very differently from those in normal medium. They failed to keratinize, and by the 7th day had become transformed into translucent, multilocular vesicles. The epithelium was much thinner than in the controls, and by the 9th day the inner border had become very refractile. At this stage ciliation was observed in the living tissue. When the first series of hanging-drop cultures in +A medium was being examined after 9 days' growth, a loose cell in the cavity of one of the cysts was seen to be rapidly spinning. The field was examined under higher magnification and two actively beating, ciliated cells were found in the epithelium near the loose cell and were responsible for its motion. In subsequent experiments ciliation was noted in five other cultures, sometimes in all the cells of one area and sometimes only in isolated, scattered cells. In one explant most of the cells lining a small cyst were beating vigorously, while in another ciliated cells and feather germs were present in the same vesicle. It is probable that more cultures contained ciliated cells than were seen in life, as

it was often impossible to obtain a clear view of the epithelium in optical section in these older explants, owing to the fairly dense fibrous capsule which formed around the cysts.

The distended vesicles in some of the +A cultures collapsed between the 9th and 14th day and the epithelium then became much thicker, and while remaining healthy towards the periphery, the innermost region of the islet was often rather degenerate.

In section, the +A cultures fixed after 9–14 day's growth in hanging-drop preparations (Pl. 2, fig. 10; Pl. 3, figs. 16, 19) presented a startling contrast to the controls in normal medium. As in the watch-glasses, keratinization was completely suppressed, and the epithelial vesicles which in the controls were flattened and irregular in cross-section, were much distended in the +A cultures and had a smooth oval shape. Besides the main vesicles, there were usually smaller cysts and pockets, some of which communicated with one of the main cavities. All these vesicles were lined by secretory epithelium which bore little resemblance to the keratinizing, squamous epidermis of the controls.

The secretory epithelium was thickest and most highly differentiated at the circumference of the main cysts and in the smaller pockets and islets (Pl. 3, fig. 19). In these sites it was usually about four cells deep; the cells of the superficial layer were cubical or columnar (Pl. 2, fig. 10) and their vacuolated cytoplasm was engorged with material which stained bright red with PAS (Pl. 3, fig. 19) or mucicarmine, pale blue with azan and deep violet with Delafield's haematoxylin and chromotrop. This substance could often be seen in course of discharge into the cavity, which contained much secretory material. In azan-stained preparations, small red granules were seen in the secretory cells, especially near their free surface. Mitosis was common, not only among the basal cells, but in all layers of the epithelium. The epithelium of the upper and lower walls of the major cysts was often very attenuated and might consist of only two flattened layers of which the inner was secreting; in other cultures it was thicker in these regions and was composed of a flattened secretory stratum below which were several layers of polyhedral cells. In general, secretion was most profuse in the equatorial zone of the large cysts and in the small, peripheral vesicles; this was probably due to better nutritive conditions in the outer than in the central part of the explants. Ciliated cells were often seen (Pl. 2, fig. 10). It was noteworthy that in the hanging-drop preparations, unlike the watch-glass cultures, the epithelial cells did not lose their cohesion but remained closely and regularly orientated.

As already mentioned, some of the vesicles collapsed about the 11th day, possibly from mechanical damage at transplantation. The consequent release of tension seemed greatly to increase the secretory activity of the cells. In some places (Pl. 3, fig. 16) deep columnar cells, stuffed with secretion, rested on a flattened stratum below which was a basal layer of cubical cells.

In a few cultures, at least some of the epithelium formed a continuous covering over all or part of the explant and zone of outgrowth; the outermost cells secreted as actively as in the cysts. Feather germs projected both from the sheets and into the vesicles, and were covered by the usual type of secretory epithelium (Pl. 3, fig. 18).

The connective tissue formed a finely fibrous capsule around the epithelial cells. Cartilage was rarely present, as it usually disappeared soon after it was formed, but in two cultures ossifying nodules persisted which showed histological changes similar to those described by Fell & Mellanby (1952).

A comparison between the secretory epithelium of the cultures in +A medium and the nasal epithelium of the normal chick

The extraordinary changes produced in the ectoderm of 6- to 7-day chick embryos by cultivation in medium containing a high concentration of vitamin A, naturally raised the question of whether the secretory epithelium into which the explanted ectoderm differentiated was histologically comparable with any tissue normally present in the body. The obvious course was to examine a secretory membrane of ectodermal origin, and the nasal mucosa was selected for the purpose. Its histogenesis was not investigated in detail and only the salient features of its structure in 10-, 13- and 18-day embryos and in the adult cock will be mentioned.

The nasal epithelium of a 10-day embryo consisted of a superficial flattened layer and a basal layer of columnar cells. On the nasal septum and elsewhere in the organ, slight thickenings had been formed at short intervals giving the epithelium a wavy appearance in section; these thickenings were the rudiments of the mucous glands but secretion had not yet begun.

In the 13-day embryo (Pl. 1, fig. 6) in most areas the epithelium had a similar appearance to that seen at 10 days, but it consisted of more than two cell layers, the depth varying in different parts of the organ, and the rudiments of the glands were more distinct and had a slight depression on the free surface, indicating the onset of invagination. There was still no sign of secretion in the septal epithelium but it had begun in some other regions. For example, in the atrium, antero-lateral to the olfactory epithelium, the superficial flattened cells had gone and had been replaced by shallow, columnar elements resting on two rather irregular layers of polyhedral cells; the free border of the columnar cells stained vividly red with PAS and red globules were usually present in the cytoplasm.

In the 18-day embryo, development had advanced greatly. The superficial flattened layer had now almost disappeared throughout the nasal epithelium and been replaced by columnar or cubical cells; the septal glands had invaginated to form pockets and were secreting quite actively (Pl. 1, fig. 8; Pl. 3, fig. 20). Their round nuclei were situated nearer the base of the cells and

the cytoplasm distal to the nucleus stained brilliantly red with PAS (Pl. 3, fig. 20) or mucicarmine and deep violet with Delafield's haematoxylin and chromotrop, but the cells were not yet distended into the adult goblet form; in preparations stained with azan, the cytoplasm was seen to contain vacuoles coloured blue and many small orange-red granules. The deep columnar epithelium between the mouths of the glands was now ciliated.

The ciliated cells of the adult nasal septum were similar to those in the 18-day embryo, but the cells of the glandular epithelium were of the typical goblet shape, with a flattened basal nucleus and cytoplasm enormously distended by mucus.

From this brief sketch of the development of the nasal epithelium in the normal fowl, it will be clear that the differentiation of the ectoderm in the +A explants of the body wall and limbs is remarkably similar. In both the nose and the +A cultures the epithelium was at first two-layered, then increased in thickness by the formation of additional layers (Pl. 1, cf. figs. 4 and 6); it lost its superficial stratum of flattened cells and these were replaced by cubical or columnar elements which became secretory and ciliated (Pl. 3, cf. figs. 16, 19 and 20), though in the cultures the flattened cells in some areas might persist and themselves secrete.

The papilliform folding of the secretory epithelium in the older watch-glass cultures may have represented an attempt to form multiple, glandular invaginations similar to those of the nasal mucosa. As stated above, gland-like ingrowths of the epithelium sometimes occurred in the explants, but it is probable that in the cultures the connective tissue was resistant to invagination on a large scale owing to its oedematous condition in the early stages of cultivation and its density after the oedema disappeared. In the cysts, the epithelium would have been held flat by the pressure of the fluid in the cavity.

The development of the secretory cells was almost identical in the two tissues; in both the secretion appeared first near the free border of the cells and then accumulated in vacuoles in the distal cytoplasm; the vacuoles had exactly the same staining reactions in the two epithelia, and the cells of both contained small granules coloured orange-red with azan.

In view of these striking resemblances, it is concluded that excess vitamin A caused the ectoderm of the trunk and limbs to differentiate into a ciliated, mucus-secreting membrane of the same histological type as that lining the nasal cavity.

II. *The effect of transferring +A explants to normal medium*

When +A explants were transferred to normal medium the following events usually took place: (*a*) at first the development of the ciliated and secretory epithelium progressed rapidly, (*b*) further formation of such cells was arrested, (*c*) the basal cells multiplied and formed a squamous epithelium beneath the

secretory layer, (*d*) eventually the ciliated, secretory epithelium was shed and degenerated.

Watch-glass cultures

Sixteen explants were grown in + A medium for 7 days. Four were then fixed, four were maintained in + A medium for 14 days and eight were transferred to normal medium, in which four were grown for 4 days and four for 8 days.

The behaviour during life of the explants grown in + A medium for 7 and 14 days has already been described (p. 474). During the first 2 days after transfer to a normal clot, the explants grew well and appeared healthier than those which remained in the + A medium; they had a very large outgrowth, the epithelium was thick and clear and the feathers were unusually well developed. By the 4th day after transfer, outgrowth was much reduced owing to advancing epithelialization of the explants, the epithelium was thinner and in places seemed to be cornifying, while the feather rudiments had become more opaque; slimy material, now known to be mucus, adhered to the surface. The oedematous condition seen at an earlier stage of cultivation, and which had later disappeared, began to return and some of the feather germs had become bloated. After 7 days in normal medium, the epithelium was almost certainly keratinizing and the oedema of the connective tissue had increased; mucus still clung to the surface of the explant.

The four explants fixed after 7 days in + A medium (Pl. 1, fig. 4) showed the same histological structure as that previously described (see p. 475). In the four sister explants fixed 4 days after transfer to normal medium, the differentiation of the secretory epithelia was much more advanced. The superficial flattened layer had largely disappeared and much of the surface was covered by regular columnar epithelium, the cells of which were often crammed with secretory material (Pl. 3, fig. 14). In all the cultures ciliated cells were present, often in large tracts which closely resembled those of the normal nasal mucosa of an 18-day embryo (Pl. 1, cf. figs. 7, 8).

Interesting changes were in progress beneath the columnar layer, and were most pronounced near the periphery of the explant. At the extreme margin where in the 7-day + A cultures, mucus-secretion was greatest, the epithelium had differentiated into the squamous type and was beginning to keratinize; on the surface of the squamous layer the secretory tissue was sometimes bundled together in an untidy vacuolated mass. Nearer the centre of the explant, the epithelium often consisted of an orderly palisade of columnar cells, while below and sharply separated from them was a newly formed squamous epithelium (Pl. 3, fig. 14). In large areas near the apex of the explant, the epithelium was entirely of the secretory type, with an outer columnar layer resting on one to two layers of polyhedral or cubical cells; mitoses were very abundant.

One culture, which had become completely epithelialized, presented a rather different appearance (Pl. 1, fig. 7). The epithelium had differentiated extensively, had formed ciliated cells and was secreting mucus, but it had become disorganized into a papilliform structure of irregular, partly detached cords and sheets. Below this was another, very irregular epithelium, which remained attached to the connective tissue. There was very little mitosis in this culture.

The squamous structure was still further developed in the epithelium of the four cultures fixed after 7 days' growth in +A medium followed by 7 days in normal medium (Pl. 2, fig. 9). Much of the secretory, ciliated tissue had been shed and was degenerating on the surface of the keratinizing epidermis forming below it, the basal cells of which were dividing very actively. The epidermal layer varied greatly in depth in the same explant. The explants were now almost completely epithelialized.

The structure of the epithelium in sister explants grown for 14 days in +A medium has already been described (p. 476); it showed no sign of squamous differentiation.

Hanging-drop preparations

Two experiments were made. In the first, seventeen explants were grown for 11 days in +A medium, when eight of them were fixed and nine transferred to normal medium; three of the nine were fixed after 5 days and six after 7 days in the normal clot. For the second experiment, nine explants were cultivated in +A medium for 14 days after which four were fixed; of the remaining five, three were grown in normal medium for 4 days and two for 8 days.

The vitamin A added to the plasma was in the form of the alcohol, which, as already mentioned, was found to be slightly toxic as compared with the acetate. In the first experiment, a concentration of 2500–2800 i.u. of vitamin A per 100 ml. plasma was used. For the first 4 days the cultures grew extremely well and the epithelium lining the vesicles looked very healthy, but by the 7th day some cell degeneration could be seen which had increased by the 11th day. When the eight cultures, fixed at this stage, were examined histologically, the epithelium had the appearance already described in section A. Secretion was fairly plentiful (Pl. 3, fig. 18), though less than in the cultures with vitamin A acetate, and in one explant a tract of ciliated cells was seen; there were a good many degenerate cells.

Two days after they had been transferred to normal medium, several of the cultures had obviously recovered and the tissue again looked healthy and vigorous but in others, though outgrowth was profuse, epithelium could no longer be distinguished. Four days after transfer, the vesicles in the recovered cultures were seen to be lined by thick, clear, obviously secretory epithelium

and in one an actively beating, ciliated cell was observed. By the 7th day in normal medium, the fibrous capsule around the cysts had become so dense that the structure of the epithelium was obscured, but in one explant it was very thick and seemed to be of the squamous type.

Of the nine cultures histologically examined after 5–7 days in normal medium, five were of no interest as the epithelium had largely disintegrated, but the remaining four were very healthy. In one fixed after 5 days in normal medium, the epithelium lining the cyst had become completely transformed into the keratinizing, squamous type and the degenerate remains of the secretory cells had been shed into the cavity. The other three contained large tracts of huge goblet cells charged with mucus which closely resembled those of the normal adult nasal mucosa (Pl. 2, fig. 11), while elsewhere in the same cyst squamous differentiation was advanced and was sometimes proceeding beneath the goblet cells (Pl. 3, fig. 17); mitosis was very plentiful. The cavities of the cysts contained much mucus.

For the second experiment a lower concentration of vitamin A alcohol was used (1920–2085 i.u./100 ml. plasma). When the living cultures were examined on the 11th day, they appeared quite healthy and in four active ciliation was observed. After 14 days some degeneration had taken place, but sections showed that the epithelium was healthier than in the previous experiment (Pl. 2, fig. 10; Pl. 3, fig. 19). On the other hand, mucus-secretion was in general less than with higher concentrations of either vitamin A acetate or alcohol, and in the deeper parts of the explant, where probably less vitamin was available, the epithelium, though not keratinized, was sometimes secreting very feebly or not at all.

Of the five cultures fixed 4–8 days after transfer to normal medium, four contained healthy epithelium, but in the fifth which was fixed owing to contamination, the epithelium had degenerated. The secretory epithelium of the two fixed after 4 days had differentiated to an astonishing degree, forming large goblet cells. Mitosis was profuse. There was no sign of squamous differentiation except possibly in one region in one explant. The two explants fixed 8 days after transfer, however, both contained areas of keratinizing squamous epithelium and in one (Pl. 2, fig. 12) a large mass of keratin had been formed which occupied about half the cavity of the cyst, the other half being filled with mucus. In both explants, active mucus-secretion was still in progress in places, but much of the secretory tissue had been shed. Mitosis was still very common in the epithelium.

DISCUSSION

Experiments on the effects of vitamin A-deficiency on animals have made it clear that this vitamin is an essential factor in the control of keratinization. In the normal animal it would seem that a certain amount of the vitamin is

required to suppress keratinization and that this minimum level varies widely for different epithelia. Thus the concentration normally present in the blood suffices to inhibit keratinization in the nasal mucosa *in vivo*, but a serious drop in this amount causes squamous metaplasia and cornification (Mori, 1922*a*, *b*; Wolbach & Howe, 1925; Jungherr, 1943). On the other hand, in explants of embryonic chick ectoderm, which is normally a keratinizing epithelium, a high level of the vitamin is required to prevent keratinization, and when the explants are returned to normal medium, keratinization ensues.

In the case of the chick ectoderm, however, we are faced by the extraordinary fact that excess vitamin A not only inhibits keratinization, but more prolonged action also causes the tissue to develop into another type of epithelium with an entirely different function from that of the epidermis into which it normally differentiates in the body. This is the reverse of the process which is produced by deficient vitamin A in animals, where the nasal and tracheal epithelia which are mucin-secreting and ciliated, are converted to the squamous keratinizing type. It may be that in the explanted ectoderm, the prevention of keratinization is the primary effect of the increased vitamin and the metaplastic effect only secondary; if so it must be assumed that the ectoderm of the trunk and limbs, being unable to follow its presumptive course of development, is deflected into an alternative path which is normally followed in the nasal region.

In recovery experiments, when the explants of chick ectoderm were transferred from +A to normal medium, the undifferentiated basal cells formed a squamous, keratinizing epithelium beneath the secretory layer. The histogenetic process was almost identical with that described by Wolbach & Howe (1925) in the squamous metaplasia of the respiratory epithelium of vitamin A-deficient rats. These authors state (p. 764): 'in the nares and trachea, mucus-secreting, ciliated epithelium with normal staining reactions and normal appearing nuclei may lie upon a keratinizing layer of cells'; some of their figures strikingly resemble those of the +A explants after transfer to normal medium (cf. Wolbach & Howe, 1925, Pl. 39, fig. 11 with our Pl. 3, fig. 14).

A curious feature of the present experiments in which the skin tissue was transplanted from +A to normal medium, was the sudden, temporary spurt in the development of the secretory epithelium during the first 4–5 days after the transfer. As described above, during this period both the ciliated and the goblet cells reached a stage of development almost equivalent to that of similar elements in the adult nasal mucosa. The reason for this is not known. The differentiation of these cells was irreversible and, as in the nasal mucosa of the vitamin A-deficient rat (Wolbach & Howe, 1925), eventually they were shed and degenerated.

The mechanism whereby different concentrations of vitamin A produce metaplasia in epithelia both in the animal and in culture, is completely

obscure, but whatever it may be it appears to act on those cells which are basic to the growth of the tissue, in this case those of the stratum germinativum (cf. Wolbach & Howe, 1925, 1933). This direct action of the vitamin on master structural cells is also seen in bone. As shown by one of us (Mellanby 1938, 1944, 1947), when vitamin A becomes deficient in young growing animals, the position and degree of activity of the osteoblasts and osteoclasts are greatly altered, the mechanism of bone modelling breaks down and the bone becomes thickened, coarse and malformed. When the vitamin A is sufficiently above normal, there is also an alteration of bone cell activity in the opposite sense, and the bones become rarified and fracture easily. Another instance of vitamin A acting on structural cells can be seen in embryonic bone growing in tissue culture. There the high vitamin A stimulates the chondroblasts to destroy the matrix of the cartilage prior to their own disappearance (Fell & Mellanby, 1952). These, of course, are effects of vitamin A on mesodermal cells in contrast to its action on ectodermal cells described in the present work. The fact that vitamin A acts on basic structural cells in both ectodermal and mesodermal tissues may be of significance, and may lead to a better understanding of this interesting biological effect of the vitamin.

Whereas there are probably different threshold values of vitamin A for controlling the structure and function of different epithelia in an animal, it is also probable that there are differences of threshold controlling the same epithelium in different species. This seems likely from the great differences of vitamin A concentration in the blood of different species. Normally rat plasma contains 50–80 i.u./100 ml., a level which would correspond to hypovitaminosis in dogs or chickens in which normal values lie between 200 and 350 i.u./100 ml., values corresponding with induced hypervitaminosis in rats. The great difficulty in producing hypervitaminosis A and the need to give massive doses of the vitamin to produce this condition, shows how important to the well-being of the animal is the mechanism of keeping the plasma concentration constant at the level normal to each species. Whereas the significance of this control is now obvious, except for the storage phenomenon in the liver, knowledge of the mechanism of control is meagre.

In view of these great differences in the normal amount of vitamin A present in the blood of different animals, it may well be that the ectoderm of species other than that of chick embryos may vary in their susceptibility to excessive vitamin A. Preliminary experiments with the early embryonic mouse ectoderm in culture, have shown that it is far less reactive than that of the chick, and while there was evidence of an inhibitory effect on hair-formation and eventually on epidermal keratinization, for the first week at least, plentiful keratin was formed in the presence of a concentration of vitamin A which completely prevented cornification in the chick cultures. Further experiments will be made with mammalian tissues.

SUMMARY

1. Explants of 6- to 7-day embryonic chick ectoderm from the trunk and limbs were cultivated *in vitro* in medium containing excess vitamin A; control explants from the opposite side of each embryo were grown in normal medium.

2. The high vitamin A completely suppressed keratinization and caused the ectoderm to differentiate into mucus-secreting, often ciliated epithelia similar to that of the normal nasal mucosa.

3. The control explants in normal medium formed keratinizing squamous epithelium.

4. When the explants were transferred from the high vitamin A medium to normal medium, the differentiation of the secreting epithelia was at first greatly accelerated and reached an advanced stage.

5. The basal cells ceased to form new secretory cells after transfer to normal medium, and instead differentiated into a squamous, keratinizing epithelium beneath the secretory layer; eventually the secretory and ciliated cells were shed.

The authors wish to express their gratitude to Dr W. Jacobson for the colour photographs in Pl. 3. They are indebted to Mr R. J. C. Stewart, chief technician at the Nutrition Building, National Institute for Medical Research, for preparing the blood plasma, to Mr V. C. Norfield, head assistant at the Strangeways Research Laboratory, for the monochrome photographs, and to Mr L. J. King, technician at the Strangeways Research Laboratory, for assistance with the tissue culture.

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EXPLANATION OF PLATES

The photographs in Pls. 1 and 2 are by Mr V. C. Norfield and those in Pl. 3 by Dr W. Jacobson. *Abbreviations: c*, cilia; *mg*, mucous gland; *n*, nucleus; *s*, secretion.

PLATE 1

Fig. 1. Control explant of 7-day embryonic skin grown in normal medium for 7 days; watch-glass culture. Note the thick, keratinized squamous epithelium. In the connective tissue there is an outer dermal layer and a more compact inner mass. Cells are migrating into the clot. ×60. (Delafield's haematoxylin; chromotrop.)

Fig. 2. Explant from the opposite side of the same embryo grown for 7 days in +A medium; watch-glass culture. Note the absence of keratinization. The connective tissue shows the same two zones as in the control but is rather oedematous. ×60. (Delafield's haematoxylin; chromotrop.)

Fig. 3. Lateral ectoderm of normal 7-day embryo. It consists of a basal columnar and a superficial flattened layer. ×500. (Azan.)

Fig. 4. Skin explant grown in +A medium for 7 days; watch-glass culture. Secretion has just begun (*s*). The epithelium resembles that of the normal nasal mucosa of a 13-day embryo (cf. fig. 6). ×500. (Periodic acid–Schiff (PAS) and Meyer's haematoxylin.)

Fig. 5. Skin explant after 14 days' cultivation in +A medium; watch-glass culture from same experiment as explant in fig. 4. The ectoderm has formed a mass of irregular cords. ×500. (Azan.)

Fig. 6. Normal mucosa from the nasal septum of a 13-day embryo. Note the rudiments of the mucous glands (*mg*). ×500. (PAS; Meyer's haematoxylin.)

Fig. 7. Skin explant grown in +A medium for 7 days and then transferred to normal medium for 4 days; watch-glass culture from same experiment as for figs. 4 and 5; note the remarkable differentiation of the ectoderm into ciliated, secretory epithelium which closely resembles that of the normal nasal mucosa of an 18-day embryo (cf. fig. 8). ×500. (PAS; Meyer's haematoxylin.)

Fig. 8. Normal mucosa from the nasal septum of an 18-day embryo, showing ciliated epithelium and an actively secreting mucous gland. ×500. (PAS; Meyer's haematoxylin.)

PLATE 2

Fig. 9. Skin explant grown in +A medium for 7 days and transferred to normal medium for 7 days; watch-glass culture from the same experiment as for figs. 4, 5 and 7. A squamous epithelium has formed beneath the ciliated, mucus-secreting epithelium which is being shed. ×500. (Azan.)

Fig. 10. Skin explant grown in +A medium for 14 days, showing part of an epithelial vesicle; hanging-drop culture. Note the cilia, which in life were seen actively beating. Another section of this explant is shown in Pl. 3, fig. 19. ×950. (Azan.)

Fig. 11. Skin explant grown for 11 days in +A medium and then transferred to normal medium for 4 days; hanging-drop culture. Note the typical goblet cells, distended with mucus and with flattened basal nuclei (*n*). On the left of the photograph squamous epithelium is beginning to form beneath the goblet cells, a process which is well advanced in other parts of the vesicle. ×700. (Azan.)

Fig. 12. Skin explant grown in +A medium for 14 days and then transferred to normal medium for 8 days; hanging-drop culture from the same experiment as for fig. 10, showing part of an epithelial cyst. Note the squamous epithelium and large amount of keratin that has been formed during cultivation in normal medium; the cyst contains mucus also and elsewhere the epithelium is still secreting. ×500. (PAS; Meyer's haematoxylin.)

PLATE 3

Fig. 13. Skin explant grown in +A medium for 14 days; watch-glass culture from the same experiment as for figs. 4, 5, 7 and 9. Note the single layer of actively secreting columnar epithelium. ×850. (Azan.)

Fig. 14. Skin explant grown in +A medium for 7 days and then transferred to normal medium for 4 days; watch-glass culture from the same experiment as for figs. 4, 5, 7, 9 and 13. Note the squamous epithelium developing beneath the secretory, ciliated layer. ×600. (PAS; Meyer's haematoxylin.)

Fig. 15. Control skin explant grown in normal medium for 11 days; part of an epithelial cyst in a hanging-drop culture. The ectoderm has differentiated into squamous keratinizing epithelium. ×380. (PAS; Meyer's haematoxylin.)

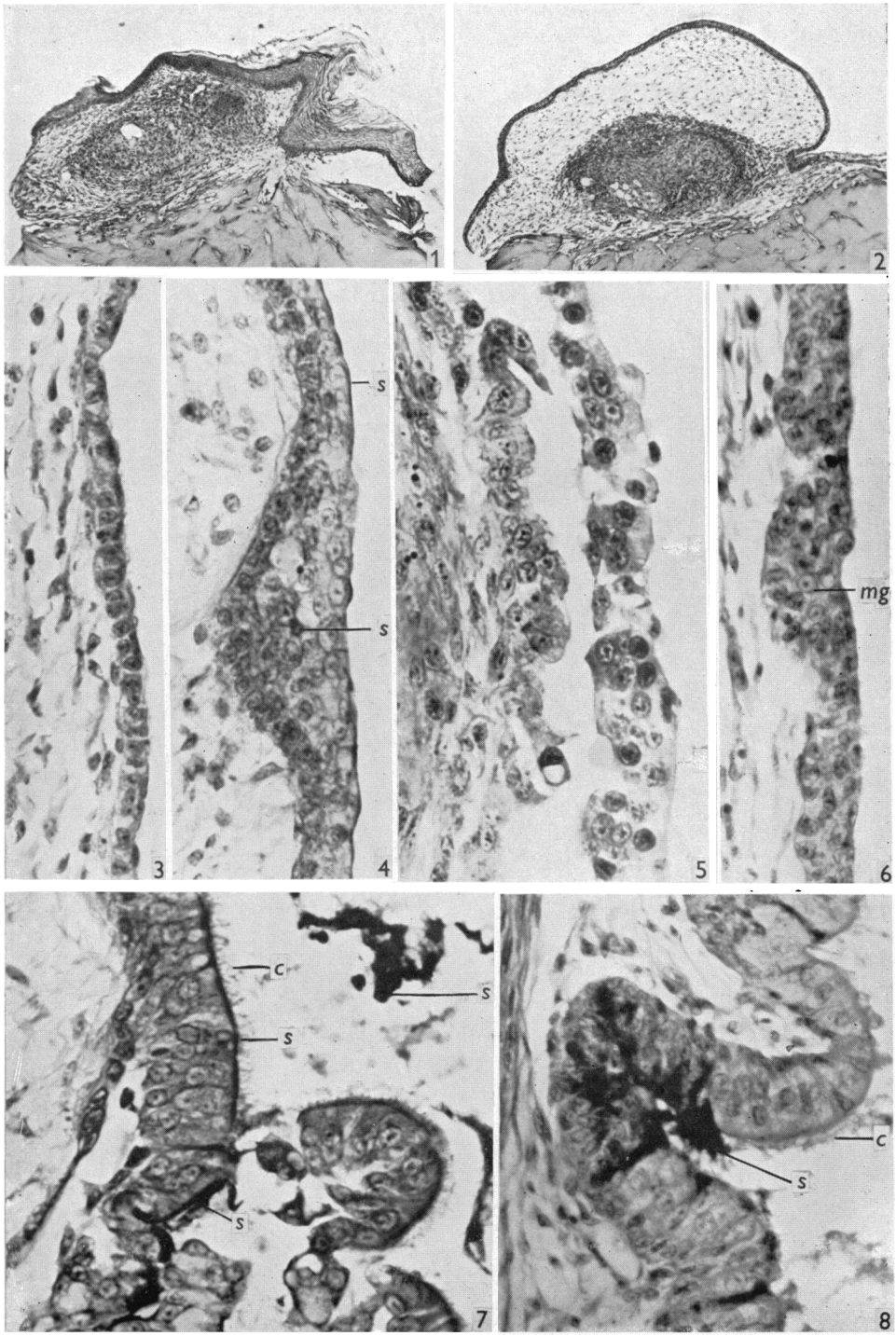
Fig. 16. Skin explant from the opposite side of the same embryo as that shown in fig. 15, after 11 days' growth in +A medium. The ectoderm has differentiated into a secretory epithelium and the cavity of the cyst is filled with mucus. ×380. (PAS; Meyer's haematoxylin.)

Fig. 17. Skin explant grown in +A medium for 11 days and then transferred to normal medium for 7 days; hanging-drop culture from the same experiment as for fig. 11. Note the squamous epithelium which is being formed beneath the goblet cells; the latter are bunched together and some are being shed. ×250. (Azan.)

Fig. 18. Skin explant grown in +A medium for 11 days; hanging-drop culture from the same experiment as for figs. 11 and 17, showing feather germ covered by secretory epithelium. ×180. (PAS; Meyer's haematoxylin.)

Fig. 19. Skin explant after 14 days' growth in +A medium; another section of the same hanging-drop culture as that shown in fig. 10. Note the secretory epithelium lining the cyst which is filled with mucus. This epithelium is ciliated (see Pl. 2, fig. 10). ×150. (PAS; Meyer's haematoxylin.)

Fig. 20. Normal nasal mucosa from the septum of an 18-day embryo, showing a mucous gland and ciliated cells. ×470. (PAS; Meyer's haematoxylin.)



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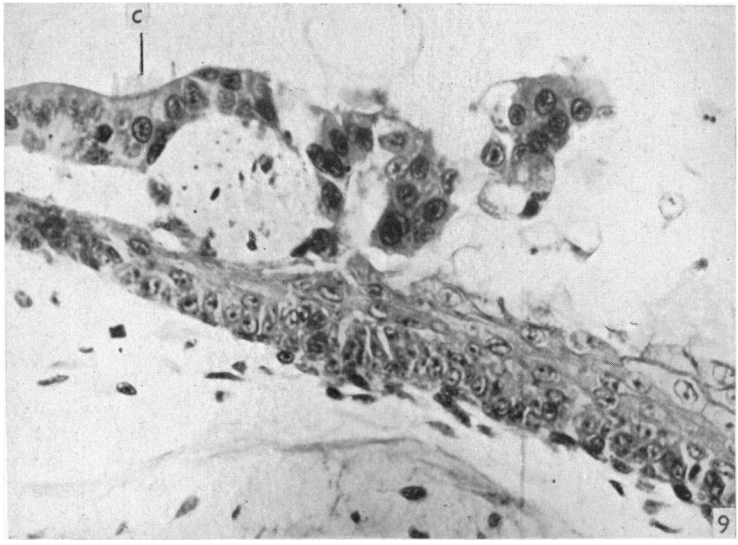
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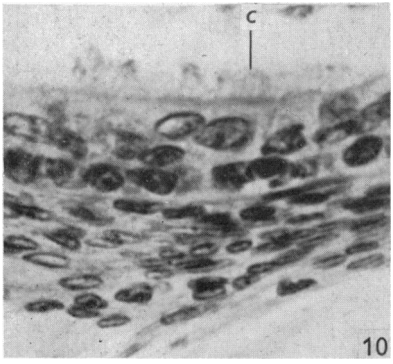
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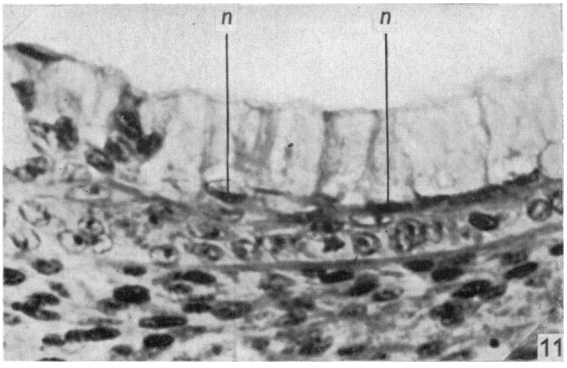
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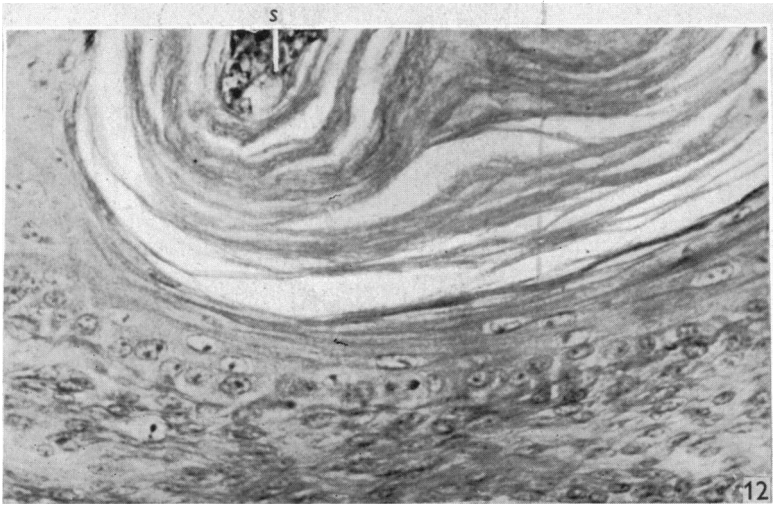
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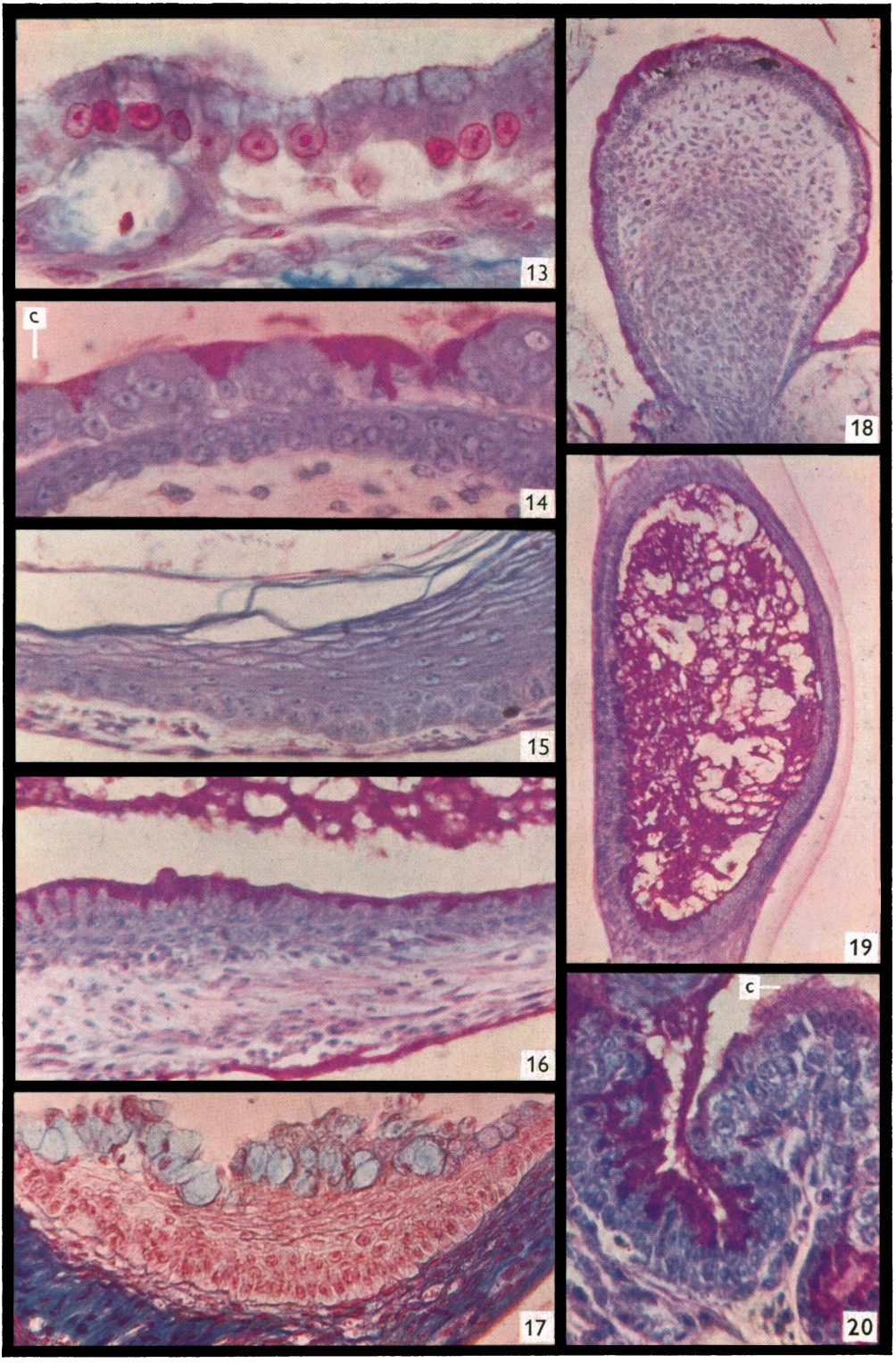
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