The Effects of Vitamin A and Citral on Epithelial Differentiation in vitro 2. The Chick Oesophageal and Corneal Epithelia and Epidermis

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WITH THREE PLATES

INTRODUCTION

The effects of vitamin A and citral on the differentiation of chick tracheal epithelium in vitro were described in a previous paper (Aydelotte, 1963a). High concentrations of vitamin A inhibited the development of tracheal mucous cells but the epithelium became well ciliated. Citral in low concentrations favoured the differentiation of mucous cells but few ciliated cells developed; in higher concentrations of citral the tracheal epithelium became stratified and occasionally keratinized. The changes produced by citral resembled those in the tracheal epithelium of vitamin A deficient chicks (Aydelotte, 1963b) and when vitamin A and citral were both added to the culture medium, the combined effect was intermediate between those given by the two compounds separately. These results, therefore, supported the suggestion put forward by Leach & Lloyd (1956) that citral inhibits vitamin A.

The investigation of the effects of vitamin A and citral in vitro has been extended to the oesophageal and corneal epithelia and epidermis of the chick embryo. These epithelia are stratified, and therefore differ from the tracheal epithelium, and, moreover, they are known to be influenced by the environmental concentration of vitamin A. In vitamin A deficient chicks, the oesophageal mucous glands atrophy as they are gradually replaced by keratinizing epithelium (Seifried, 1930; Aydelotte, 1963b). The chick corneal epithelium, normally smooth, stratified and squamous, becomes slightly thicker and rough, whilst the adjacent conjunctival epithelium changes from a thin, secretory membrane to a thick, keratinized layer (Beach, 1923; Aydelotte, 1963b). Keratinized epidermis, on the other hand, is relatively little affected by deficiency

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of vitamin A, but it has been clearly shown that high concentrations of vitamin A inhibit the normal keratinization of embryonic chick skin in culture and induce mucous differentiation (Fell & Mellanby, 1953; Fell, 1957). This metaplastic change is the reverse of that which occurs in many mucous membranes (e.g. the tracheal epithelium) in deficiency of vitamin A. It seemed likely that high concentrations of vitamin A might also alter the differentiation of the stratified oesophageal and corneal epithelia in vitro.

If citral inhibits the responses of these epithelia to high concentrations of vitamin A, this would be valuable additional evidence that citral is an antagonist of the vitamin.

Cultures of chick oesophagus, cornea and skin were grown in normal medium and in media containing additional vitamin A and/or citral. Both compounds altered the pattern of differentiation of the oesophageal and corneal epithelia, and the results of these experiments support the view that citral is an inhibitor of vitamin A.

MATERIALS AND METHODS

Culture method

Explants, supported by pieces of cellulose-acetate net (Shaffer, 1956), were grown on a semi-solid medium in sealed embryological watch-glasses. The basic culture medium consisted of 50 per cent. fresh cock plasma, 25 per cent. Tyrode's solution and 25 per cent. chick embryo extract (prepared from equal volumes of minced 13-day embryo and Tyrode's solution). Antibiotics were not used. For experimental media, vitamin A (synthetic crystalline vitamin A alcohol, prepared by Eastman Kodak Co., U.S.A.) and/or citral (B.D.H. laboratory chemical) were dissolved in ethanol and added to the plasma before making up the media. An equal amount of ethanol was added to the plasma for the control cultures, but the final concentration of ethanol in the medium never exceeded 0·15 per cent.

The organs required for culturing were dissected from 13-day chick embryos and placed in sterile Tyrode's solution. Under a dissecting microscope, loose connective tissue was cleaned away and the organs were cut into pieces suitable for explantation. The upper oesophagus (i.e. the part above the crop) was opened by one longitudinal incision and then divided into three or four lengths. The cornea was explanted whole, sometimes with a narrow rim of attached conjunctiva. Skin from the shank of the leg was carefully separated from the underlying muscle and cut into pieces roughly 2×3 mm. in size.

The explants were transferred to pieces of rayon net moistened with Tyrode's solution, and were orientated with the epithelium uppermost and the connective tissue in contact with the rayon and culture medium. The cultures were incubated at 38°C. and were washed and transferred to fresh medium every 2 days.

In each experiment the explants were divided into four groups and cultured on the following media: (1) Normal; (2) + Vitamin A; (3) + Citral; (4) +
Vitamin A + citral. Oesophageal explants were cultured for periods ranging from 4–12 days, corneal explants from 6–10 days and epidermis from 6–12 days.

_Histology_

At the end of the culture period the oesophageal and corneal explants were fixed in Zenker-formol, and skin explants in 3 per cent. acetic-Zenker for 30 min., followed by Zenker without acetic acid for 1½ hr. (Fell, 1957). The cultures were embedded in paraffin wax and serial sections 5 μ in thickness were stained with Mayer's acid haemalum and alcian blue, and with periodic acid Schiff and haemalum. Material for comparison with the cultures was dissected from the embryos or young chicks, washed briefly in Tyrode's solution, and fixed, embedded, sectioned and stained in the same way as the explants. One hundred and twenty-four cultures of oesophagus, forty-four of cornea and forty-four of chick skin were examined histologically.

**RESULTS**

**Oesophagus**

_Normal development in vivo_

In a 13-day embryo the oesophageal epithelium is two-layered, but 8 days later at the time of hatching it is much thicker and rapidly becoming like the stratified, squamous, non-keratinized epithelium of the adult bird. During this period of rapid growth and differentiation the surface of the epithelium becomes transiently ciliated, and mucous glands develop in the lamina propria. In a 12- or 13-day chick embryo, the first cuboidal ciliated cells appear singly or in small groups, sunk into the surface of the epithelium among the squamous cells. The ciliated cells reach their maximum number at 19 days of incubation and disappear completely a day or two after hatching. The rudimentary mucous glands first appear as solid epithelial buds after 13–15 days; as they increase in size, the buds grow down into the lamina propria and develop small vesicles which gradually fuse to form the lumina of the glands. The glands open to the oesophageal lumen at 20–21 days of incubation through a duct lined by low cuboidal mucous cells; the glandular cells at this stage are filled with mucopolysaccharides and are secreting vigorously. During the first few days after hatching, the oesophageal epithelium continues to increase in thickness and the acini of the mucous glands branch further.

_Development in culture_

_Normal medium._ Slight ciliation could occasionally be detected in a 13-day embryonic oesophagus at the time of explantation, but it was more commonly seen after 2 days _in vitro_, was fairly widespread after 6 days, and thereafter
declined slowly. Developing glands were visible as clear patches in the longitudinal furrows of the explant after 2–3 days in culture. After 6 days the explants appeared thicker and more opaque with clumps of sloughed cells and secreted mucus over the surface. Secretion reached a maximum about the 12th day in vitro and then declined fairly rapidly.

Histological examination of the explants showed that during the first 6 days in vitro development of the oesophageal epithelium was similar to that in the body, although some differences could be detected. In the normal medium, a much higher proportion of the epithelium became ciliated than in vivo. After 4 days in culture the cuboidal ciliated cells formed a distinct superficial layer over the stratified epithelium, but 2 days later this ciliated sheet was being sloughed as the underlying cells became vacuolated and loose (Plate 1, Fig. A). The mucous glands developed fairly well although they failed to penetrate as deeply as in the body.

When explants were grown for more than 6 days, development gradually deviated further from normal: many superficial layers of distended, vacuolated cells were shed leaving a more compact epithelium eight to nine layers thick after 12 days. The glands, instead of remaining deep in the lamina propria, became open, shallow pits, extending only a short distance into the connective tissue, and with mucous cells spreading over the surface of the explant, between the sloughed layers and the more compact epithelium (Plate 1, Fig. B).

*Vitamin A.* Oesophageal cultures were grown in concentrations of vitamin A ranging from 2.5–10.0 i.u./ml. Examination of living explants in +A medium showed that the mucous glands developed earlier and were more numerous than in the controls: they began to form after 1–2 days in +A medium, and after 5–6 days were scattered over the whole surface making the epithelium

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**EXPLANATION OF PLATES**

The figures are photomicrographs of sections stained with Mayer's acid haemalum and alcian blue; mucus appears dark.

**PLATE 1**

**Fig. A.** Section of an oesophageal explant, cultured for 6 days on a normal medium. Note the superficial sheet of darkly-stained ciliated cells that is being sloughed.

**Fig. B.** Section of an oesophageal explant, cultured for 12 days on a normal medium. The shallow mucous glands are spreading over the surface of the stratified epithelium.

**Fig. C.** Section of an oesophageal explant, cultured for 6 days on a medium containing 7.5 i.u./ml. of vitamin A. The epithelium is composed of tall, columnar, ciliated cells and a few mucous cells. Parts of mucous glands can be seen in the connective tissue at each side of the figure.

**Fig. D.** Section of an oesophageal explant, cultured for 12 days on a medium containing 5 i.u./ml. of vitamin A. The epithelium is stratified and contains many small mucous glands.

**Fig. E.** Section of an oesophageal explant, cultured for 12 days on a medium containing 2.0 mM. citral. The epithelium is devoid of mucous glands and is much thicker than that of the control explant shown in Fig. B.
appear folded and pitted. The + A explants remained thin and translucent and ciliation was more widespread than in the controls.

Histological examination confirmed these observations: the epithelium of the + A cultures remained thinner than that of the controls, and the majority of the superficial cells became tall, columnar and ciliated, in contrast with the cuboidal ciliated cells that developed in the controls. Vitamin A also favoured the development of superficial mucous cells, particularly at the thin edges of the explants. The shallow pits in the epithelium contained many cells that were beginning to secrete mucus, and the deeper glands were opened wider than in the control explants although they were secreting little. The most striking feature in a culture grown for 6 days on a medium containing 7.5 i.u./ml of vitamin A was the pseudostratified, folded epithelium of columnar ciliated cells (Plate 1, Fig. C).

Oesophageal explants which were grown for 12 days in a medium containing 5 i.u./ml of vitamin A were well ciliated and contained many mucous glands, although these frequently secreted less mucus than the glands of the control explants (Plate 1, Fig. D).

Thus vitamin A inhibited the normal development of a thick, stratified oesophageal epithelium but favoured the differentiation of a pseudostratified epithelium of high, columnar, ciliated and mucous cells. Although vitamin A stimulated the development of glands and mucous cells, high concentrations partially inhibited synthesis and secretion of mucus.

*Citral.* Explants were grown in medium containing 0.2-3.0 mM citral. After 4 days the oesophageal explants showed no ciliation and little or no glandular development: they gradually became opaque and appeared thicker than the control cultures of similar age.

Histological examination showed that citral almost completely inhibited the differentiation of ciliated cells and the superficial cells became squamous. A few mucous cells developed in these cultures after 6 days, but they were much less numerous than in the controls and glands were rarely formed. After 10 days in a medium containing 2.0 mM citral, the oesophageal epithelium was

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**Plate 2**

Fig. F. Section of an oesophageal explant, cultured for 12 days on a medium containing 5 i.u./ml of vitamin A and 2.0 mM citral. The explant shows a mild vitamin A effect. Compare with Figs. B, D and E.

Fig. G. Section of a corneal explant, cultured for 6 days on a normal medium.

Fig. H. Section of a corneal explant, cultured for 10 days on a medium containing 10 i.u./ml of vitamin A. The superficial cells are secreting mucus.

Fig. I. Section of a corneal explant, cultured for 6 days on a medium containing 2.0 mM citral. The epithelium is a little thicker than that of the control explant shown in Fig. G.

Fig. J. Section of a corneal explant, cultured for 10 days on a medium containing 10 i.u./ml of vitamin A and 2.5 mM citral. The superficial cells are secreting vigorously in response to the vitamin.
very thick and much more compact than that of the control explants. A few
groups of large mucous cells had developed on the surface of the epithelium
above the gland regions, but these were being replaced rapidly from below by
non-secretory cells. After 12 days in vitro some superficial cells were being
sloughed but the epithelium remained much thicker than that of the controls
(Plate 1, Fig. E).

Thus citral severely affected the differentiation of the oesophageal epithelium.
Inhibition of the development of ciliated cells was one of the earliest effects,
and could be detected at concentrations as low as 0·2 mM. Development of
mucous glands was partially inhibited by 2·0 mM. citral, and on prolonged
treatment the rudimentary glands degenerated completely and were replaced
by non-secretory cells. Citral apparently prevented the differentiation of large
numbers of mucous cells, but it did not inhibit synthesis in those mucous cells
that did develop.

Vitamin A + citral. The differences observed between +A + citral cultures
and those grown on normal medium varied according to the relative concen-
trations of vitamin A and citral. In most experiments the explants remained
translucent and showed good glandular development as in +A medium; in
one experiment, however, they become opaque, secreted little mucus and
resembled those grown in low concentrations of citral. The results of these
experiments are summarized in Table 1.

Oesophagus grown in a medium containing 2·0 mM. citral and 10 i.u./ml.
of vitamin A developed a stratified epithelium with superficial cuboidal ciliated
cells, and showed better glandular development than the control explants.
These cultures resembled those grown in a medium containing a concentration
of vitamin A lower than 10 i.u./ml. Thus in this experiment a mild vitamin A
effect was produced, but citral lowered the effective concentration of the added
vitamin. Similarly, cultures grown in a medium containing 2·0 mM. citral and
5 i.u./ml. of vitamin A showed a slight vitamin A effect, like that produced by
a lower concentration of vitamin A alone (Plate 2, Fig. F).

In one experiment, by using a relatively low concentration of vitamin A
(2·5 i.u./ml.) with a high concentration of citral (3·0 mM.), a citral effect was
PLATE 3

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(Facing p. 626)
produced: no ciliated cells or glands developed but the explants were much healthier than those grown in the medium containing the same concentration of citral alone. Thus vitamin A lowered the toxicity of citral, but did not completely suppress its effects at this concentration.

### TABLE 1

**A summary of the combined effects of vitamin A and citral in vitro**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Concentrations in medium</th>
<th>Effect</th>
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<tbody>
<tr>
<td></td>
<td>A (i.u./ml.) Citral (mM.)</td>
<td>A</td>
</tr>
<tr>
<td>Trachea*</td>
<td>2.5 3.0</td>
<td>++ +</td>
</tr>
<tr>
<td></td>
<td>10.0 2.5</td>
<td>++</td>
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<td></td>
<td>10.0 2.0</td>
<td>+</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>2.5 3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0 2.0</td>
<td></td>
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<tr>
<td></td>
<td>10.0 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0 2.0</td>
<td></td>
</tr>
<tr>
<td>Cornea</td>
<td>5.0 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0 2.5</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>5.0 1.5</td>
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<td></td>
<td>10.0 2.0</td>
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* The results of the tracheal experiments are described in an earlier paper (Aydelotte, 1963a).

**Cornea**

**Normal development** in vivo

At 13 days the corneal epithelium consists of two layers of actively dividing cuboidal cells and a superficial layer of squamous cells. The epithelium rapidly increases in thickness and by 21 days it is almost fully developed with tall columnar basal cells, several intermediate layers of polygonal to flattened cells, and superficial squamous cells. Goblet cells develop in the thin conjunctival epithelium about the time of hatching.

**Development in culture**

**Normal medium.** The cornea appeared healthy and translucent and showed very little change during the 10-day culture period. Histologically, development was similar to that in the body: after 6 days *in vitro* the epithelium was three to four layers thick in the central region (Plate 2, Fig. G), whilst at a corresponding age *in vivo* it was four to five cells thick. The basal cells of the cultured epithelium were neither as tall nor as regular in arrangement as those of the intact cornea, but these differences were probably a result of migration from the edges of the explant. Mitotic activity gradually declined in culture so that the epithelium rarely reached its full thickness. Mucous cells differentiated in the conjunctival epithelium at the edges of the explants.

**Vitamin A.** Corneal explants grown in media containing 5–10 i.u./ml. of additional vitamin A gave a more profuse fibroblastic outgrowth than the
controls, but otherwise they appeared similar. When the +A explants were changed to fresh medium after 6 days, each was covered by a thin film of mucus.

In +A medium the corneal epithelium remained much thinner than usual and the superficial cells became cuboidal and mucus-secreting (Plate 2, Fig. H). The majority of these secretory cells contained very little mucin, but fairly large quantities of secreted mucus streamed away from them and covered the whole explant. The conjunctival goblet cells at the edges of some explants also contributed to this secretion.

Counts of colchicine-blocked mitoses in the corneal epithelium suggested that the mitotic rate was lower in the +A cultures than in the controls. In one experiment the number of mitotic figures in a cornea grown in a medium containing 5 i.u./ml. of vitamin A was only 10 per cent. of that in the control explant, and in another experiment a culture treated with 10 i.u./ml. of vitamin A showed no epithelial mitoses, although the control explants had a normal number. This difference in mitotic rate between the control and +A cultures may be sufficient to account for the difference in thickness of the epithelia in the two cases.

Citral. Corneal cultures grown in medium containing 2·0 mM. citral became increasingly opaque during the culture period and the outgrowth of fibroblasts was inhibited. After 6 days in + citral medium the corneal epithelium was slightly thicker than that of the corresponding control cultures: it comprised a columnar basal layer, three intermediate layers and superficial squamous cells (Plate 2, Fig. 1). At the edges of some explants the conjunctiva was devoid of mucous cells and it became a typical keratinizing epithelium. Mitoses were numerous in the basal cells during the first 6 days in vitro, and in one experiment the mitotic rate was much higher than in the control explants. Citral apparently stimulated the division of the basal cells of the corneal and conjunctival epithelia and inhibited the differentiation of conjunctival mucous cells.

Vitamin A + citral. In some experiments no differences could be detected between the living cultures in control medium and those in + A + citral medium, but in other experiments the explants in + A + citral medium appeared slightly more translucent and secreted more mucus than the controls.

The explants showed a vitamin A effect in all these experiments (see Table 1). The epithelium of the +A + citral cultures was thinner than that of the controls and the superficial cells were low cuboidal mucous cells. The epithelium differed from that of the corresponding +A culture, however, and resembled that of a culture grown in a medium containing a lower concentration of vitamin A alone. Thus in one experiment with a medium containing 5 i.u./ml. of vitamin A and 2·0 mM. citral, the superficial cells were more flattened and secreted less mucus than those of an explant grown in the presence of 5 i.u./ml. of vitamin A alone; the epithelium was intermediate in these respects between that of the control and that of the +A explants. In another experiment, with 10 i.u./ml. of vitamin A and 2·5 mM. citral, the explants secreted more mucus
than those in the medium containing 10 i.u./ml. of vitamin A alone (Plate 2, Fig. J). Mucus-secretion was usually greatest in media containing only a small amount of additional vitamin A, higher concentrations partially inhibiting secretion.

**Epidermis**

*Normal development in vivo*

In a 13-day embryo the epidermis of the shank possesses fairly well defined scales and comprises a layer of columnar basal cells, two to three intermediate layers and two layers of flattened periderm. By the time of hatching the periderm has been sloughed and the underlying cells transformed into a thick layer of keratin.

*Development in culture*

*Normal medium.* It has been shown (Fell, 1957) that explants of chicken skin taken from the shank of 13-day embryos keratinize readily when grown by the organ culture method. In the present experiments, after 9 days the periderm was being sloughed, and the scales were well developed and covered by a keratinized epithelium of approximately the same thickness as that of the newly-hatched chick (Plate 3, Fig. K).

*Vitamin A.* In the presence of high concentrations of vitamin A keratinization is inhibited and the epithelium becomes mucus-secreting (Fell, 1957). Similar results have been obtained in the present experiments. When skin was cultured in medium containing 5 i.u./ml. of additional vitamin A, keratinization was only partially suppressed and after 6 days a very thin layer of keratin was beginning to develop in the central parts of the explants. After 9 days most of the epithelium was thin and non-keratinized and the superficial cells were synthesizing and secreting mucus (Plate 3, Fig. L), but islands of keratinized epithelium corresponding to the thick scales could be found in the central parts of the explant. This keratin was being shed rapidly and after 12 days the epithelium beneath the sloughed keratin was thinner and secretory. In a medium containing a higher concentration of vitamin A (10 i.u./ml.) keratinization was completely suppressed and the epithelium remained thin and became secretory.

*Citral.* The living explants of 13-day embryonic epidermis grown in medium containing 1·5 or 2·0 mM. citral appeared identical with the controls in normal medium; it was also difficult to detect any differences between stained sections of the two types of explant. The epithelium of the + citral cultures was healthy and had attained the same thickness and degree of keratinization as that of the controls (Plate 3, Fig. M). The keratin was sometimes more compact in the + citral skin than in the controls, but otherwise citral had no obvious effect.

*Vitamin A + citral.* Most of the explants of chick skin grown on a medium
containing 5 i.u./ml. of vitamin A and 1·5 mM. citral appeared identical with
the controls and +citral cultures. In those explants that differed slightly, the
scales appeared normal in the central regions, but slightly indistinct near the
edges.

These observations were confirmed histologically: most cultures were like
those grown in normal medium or with citral alone (Plate 3, Fig. N). In one
culture fixed after 9 days and another grown for 12 days, however, the non-
keratinized peripheral zone of epithelium was slightly broader than in the
+ citral or control cultures, and in these regions distended cells with enlarged
nuclei were being sloughed from the surface. This may have represented a very
mild vitamin A effect, but similar differentiation has often been observed at the
edges of cultures grown in normal medium. Nevertheless, it is quite clear that
citral inhibited the normal response of the epithelium to this concentration of
added vitamin A.

Cultures grown in medium containing 10 i.u./ml. of vitamin A and 2·0 mM.
citral showed a very mild vitamin A effect (see Table 1). After 6 days keratiniza-
tion of the scales in the central parts was very slightly retarded by comparison
with that in the control and +citral cultures. At the edges of the explants, the
non-keratinized zones were clearly wider than in the control and + citral cultures,
but the epithelium in these regions was much thicker than in the +A cultures
and the superficial cells were not secretory. Thus in this experiment the vitamin
A effect was much reduced, but not completely overcome by the citral; a similar
histological picture could have been obtained by using a much lower concen-
tration of vitamin A alone.

DISCUSSION

In 1956 Leach & Lloyd suggested that citral might be an inhibitor of vitamin
A. They found that citral caused endothelial damage in rabbits and monkeys
but that vitamin A could protect against and reverse this effect. Experiments
on the differentiation of chick tracheal epithelium in culture gave further
evidence in support of this theory (Aydelotte, 1963a): citral produced changes
that resembled those of vitamin A deficiency in the tracheal epithelium in vivo
but were completely opposite to those of additional vitamin A in vitro. When
the two compounds were tested together on the tracheal epithelium, the vitamin
gave partial protection against citral and a mild citral effect was produced.

The results described in the present paper also indicate that citral inhibits
vitamin A. In the oesophageal and corneal epithelia citral alone caused changes
like those of vitamin A deficiency in vivo and opposite to those of additional
vitamin A. When both compounds were added to the medium citral reduced
the responses to the vitamin.

It had been shown previously that high concentrations of vitamin A inhibited
the normal keratinization and stratification of embryonic chick epidermis in vitro and induced the development of a mucus-secreting epithelium (Fell &
Mellanby, 1953; Fell, 1957). The present experiments gave similar results. Citral, however, had no obvious effect on the developing chick epidermis. Since epidermis is hardly affected by vitamin A deficiency \textit{in vivo}, little change would be expected in these \textit{+} citral cultures, if citral acts by inhibiting vitamin A. When cultures of epidermis were exposed to both compounds simultaneously, however, citral almost completely inhibited the response to the high concentrations of vitamin A. In one experiment it was very difficult to detect any net effect of the vitamin and citral. In another experiment, although the central parts of the explants were almost normal, the extreme edges failed to keratinize; treatment with a much lower dose of the vitamin alone would have provoked a similar response.

The results of this investigation give further evidence that citral inhibits vitamin A, but the mechanism of inhibition is not yet understood. Antagonism between vitamin A and hydrocortisone was recently demonstrated in cultures of embryonic chick skin (Fell, 1962) and chick and mouse limb-bone rudiments (Fell & Thomas, 1961). Hydrocortisone alone caused precocious keratinization of skin. When it was used with additional vitamin A, the hormone at first predominated and large areas of epithelium keratinized, but later the keratin was sloughed and mucous cells, typical of a vitamin A effect, developed. In cultures of chick cartilaginous rudiments the hormone delayed, but never completely arrested, the action of vitamin A. This antagonism between vitamin A and hydrocortisone differs from that between the vitamin and citral. In the former the cells at first responded to the hormone, but eventually vitamin A always predominated. With vitamin A and citral, however, there was no evidence of a similar change in direction of differentiation during the course of any experiment, but from the first the combined effect was that of either the vitamin or citral. Occasionally the two compounds were so closely balanced that the explants appeared virtually normal.

It is not known how hydrocortisone and citral antagonize vitamin A, but Fell (1962) suggested that the hormone and vitamin might compete within the cells. Lysosomal proteases which cause dissolution of cartilaginous matrix may be released from chondrocytes by vitamin A (Dingle, Lucy & Fell, 1961; Lucy, Dingle & Fell, 1961). Possibly hydrocortisone retards the normal action of vitamin A on cartilage by delaying this release of proteases (Fell & Thomas, 1961). Fell (1962) further suggested that in cultures of chick skin, hydrocortisone might also inhibit the release of proteases by vitamin A. Antagonism of this type within the cells could explain the results observed with vitamin A and hydrocortisone if the vitamin were more stable and could accumulate and act for a longer time than the hormone (Fell, 1962). The antagonism between vitamin A and citral, however, seems more direct. Citral appears to inhibit a certain quantity of vitamin A in the medium, so that differentiation depends upon the concentration of the remaining active vitamin. Possibly citral blocks the entry of vitamin A to the cells or competes for active sites on the cell
membrane. This type of inhibition could result from competition arising from the chemical similarity between vitamin A and citral (Leach & Lloyd, 1956; Aydelotte, 1963a).

If citral makes the culture medium deficient in vitamin A, this gives a ready method of studying differentiation over a wide range of concentrations of vitamin A. Several interesting observations have been made which may have some bearing on the mode of action of vitamin A in epithelia.

It has been suggested that vitamin A affects the mitotic rate in epithelia. Thus the tracheal basal cells normally have a low mitotic rate, but with vitamin A deficiency and citral treatment the frequency of mitoses increased and, as a result, the epithelium became stratified (Aydelotte, 1963a, b). Further evidence was seen in corneal cultures: the basal cells of the epithelium showed fewer mitoses in +A medium than in control or +citral media, and in high concentrations of vitamin A the epithelium consequently remained thin, but with vitamin A deficiency or citral treatment it became thicker than normal. Similar changes were noticeable in the conjunctival epithelium which became thick and keratinized as a result of rapid cell division under the influence of citral or vitamin A deficiency. High concentrations of vitamin A, therefore, seem to inhibit mitosis in these epithelia.

Vitamin A obviously influences mucus-synthesis, and certain epithelia that do not normally secrete mucus become secretory when exposed to high concentrations of the vitamin. In the trachea, however, high concentrations of vitamin A inhibited the development of mucous cells (Aydelotte, 1963a) and it was suggested that mucus could be synthesized in this epithelium only over a limited range of concentrations of vitamin A. In the oesophageal and corneal epithelia, too, the results indicated that mucus-secretion was greatest at a particular concentration of vitamin A and that higher levels inhibited synthesis and secretion. Fell & Mellanby (1953) and Fell (1957) also found that high concentrations of vitamin A were not entirely favourable to the mucous cells that developed in chick skin cultures: secretion was usually most abundant when the metaplastic cultures were returned from the high vitamin A to the normal medium. However, the concentrations of vitamin A that were inhibitory to mucous cells in the oesophageal and corneal epithelia and epidermis were significantly higher than those that inhibited secretion in the trachea. Each epithelium seems capable of synthesizing mucus only within a characteristic range of concentrations of vitamin A.

Since epithelia vary in sensitivity to high concentrations of vitamin A, they also vary in sensitivity to deficiency of the vitamin and to citral. Secretory epithelia are damaged earliest and most severely by vitamin A deficiency and are likely to be the most sensitive to citral. Stratified epithelia, on the other hand, show marked changes with vitamin A treatment and are probably more sensitive to additional vitamin A than are simple secretory epithelia. Such considerations may explain why certain concentrations of vitamin A and citral
produced a vitamin A effect in one epithelium and a citral effect in another. In the tracheal epithelium citral completely suppressed the vitamin (Aydelotte, 1963a), whereas in the other epithelia vitamin A usually predominated (see Table 1).

SUMMARY

1. Differentiation of the oesophageal and corneal epithelia and epidermis of the chick embryo was studied in organ cultures in normal medium and in media containing added vitamin A and/or citral.

2. In normal medium the oesophageal epithelium developed well for the first 6 days, but between 6 and 12 days many superficial layers of cells were sloughed and large mucous cells from the glands spread over the remaining epithelium. Vitamin A inhibited the normal stratification, and the oesophageal epithelium became pseudostratified and folded, with tall, columnar, ciliated cells and many small mucous glands. Citral inhibited the differentiation of ciliated and mucous cells and the epithelium became thicker than in normal medium. Vitamin A and citral together produced either a vitamin A or a citral effect, depending upon the relative concentrations of the two compounds.

3. The corneal epithelium differentiated normally in the control cultures, but under the influence of additional vitamin A it remained thin and the superficial cells secreted mucus. In + citral medium the corneal epithelium became thicker than usual and the conjunctival epithelium keratinized. When both vitamin A and citral were added to the medium the corneal cultures showed a mild vitamin A effect.

4. Skin cultures keratinized well in normal medium, but the epithelium remained thin and became mucus-secreting in response to high concentrations of vitamin A. Citral alone had little effect on epidermis, but it suppressed the vitamin A response almost completely when both compounds were added together.

5. Citral produced changes resembling those of vitamin A deficiency in cultures of oesophagus and cornea, and it reduced the effects of added vitamin A. The results of these experiments therefore give further evidence of antagonism between vitamin A and citral.

6. It is suggested that either deficiency of vitamin A or treatment with citral stimulates, whilst high concentrations of vitamin A inhibit, mitosis in the corneal epithelium.

7. The results of these experiments indicate that each epithelium achieves its maximal synthesis of mucus at a characteristic concentration of vitamin A.

RÉSUMÉ

Les effets de la vitamine A et du citral sur la différenciation épithéliale in vitro.

2. Les épithéliums oesophageen et corneen et l'épiderme, chez l'embryon de Poulet

1. La différenciation de ces tissus a été étudiée en culture d'organes, en milieu normal et dans des milieux contenant de la vitamine A et/ou du citral surajoutés.
2. En milieu normal, l'épithélium oesophagien s'est bien développé pendant les six premiers jours, mais beaucoup de couches superficielles de cellules ont été éliminées entre le 6e et le 12e jour, et de grandes cellules muqueuses provenant des glandes se sont étalées sur l'épithélium restant. La vitamine A a inhibé la stratification normale, et l'épithélium oesophagien est devenu pseudo-stratifié et plissé, avec de grandes cellules columnaires ciliées et de nombreuses petites glandes muqueuses. Le citral a inhibé la différenciation des cellules ciliées et muqueuses et l'épithélium est devenu plus épais que dans le milieu normal. La vitamine A et le citral réunis ont produit des effets soit du type vitamine A, soit du type citral, selon les concentrations relatives des deux composés.

3. L'épithélium cornéen s'est différencié normalement dans les cultures témoins, mais il est devenu mince sous l'influence de la vitamine A et les cellules superficielles ont secreté du mucus. Dans le milieu contenant du citral, l'épithélium cornéen est devenu plus épais que la normale et l'épithélium conjonctif s'est kératinisé. Quand on a ajouté au milieu à la fois de la vitamine A et du citral, les cultures cornéennes ont montré une action vitaminique adoucie.

4. Les cultures de peau se sont bien kératinisées en milieu normal, mais l'épithélium est resté mince et a sécrété du mucus, par réaction à l'égard des concentrations élevées en vitamine A. Le citral seul a eu peu d'effet sur l'épiderme, mais a presque complètement supprimé la réaction à la vitamine A quand les deux substances ont été ajoutées ensemble.

5. Le citral a produit des modifications ressemblant à celles que provoque la déficience en vitamine A dans les cultures d'oesophage et de cornée, et a diminué les effets de la vitamine A ajoutée. Les résultats de ces expériences expriment ainsi de nouveau l'antagonisme entre vitamine A et citral.

6. On suppose que la mitose est stimulée dans l'épithélium cornéen soit par la déficience en vitamine A, soit par le traitement au citral, tandis que des concentrations élevées de vitamine A l'inhibent.

7. Les résultats de ces expériences indiquent que chaque épithélium réalise sa synthèse maximale de mucus pour une concentration caractéristique de vitamine A.

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