# THE SUSCEPTIBILITY OF CHICK EMBRYO SKIN ORGAN CULTURES TO INFLUENZA VIRUS FOLLOWING EXCESS VITAMIN A

By J. S. HUANG,* Sc.D., and F. B. BANG, M.D.

*(From the Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore)*

Plates 9 to 13

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While the tissue specificity which is characteristic of virus activity in the intact host can be only partially reproduced in tissue culture (1), organ culture methods which retain some of the differentiated functions of the *in vivo* tissues seem particularly adapted to an exploration of specificity (2–4). In this study we report the increased susceptibility of organ cultures of chick squamous epithelium to influenza virus after such cultures were converted to mucous-secreting epithelium following treatment with excess amounts of vitamin A (5, 6). The tissues at the same time lost their susceptibility to vaccinia virus.

## *Materials and Methods*

*Organ Culture.*—Organ cultures of chick embryonic epidermis were prepared by the watch glass procedure (7): skin fragments from the metatarsus of 12-day chick embryos were cut into 4 to 5 mm explants, washed twice in warm Hanks' balanced salt solution (BSS), and placed epidermis side up on 6 to 7 mm rayon strips (8) which rested on the surface of clotted plasma medium in a watch-glass. The watch-glass was contained inside a Petri dish carpeted with a ring of moistened cotton, and the whole setup was incubated at 36°C. Each watch-glass contained two pieces of tissue. The cultures were transferred every 2 days to fresh medium.

*Culture Medium.*—Three parts of adult chicken plasma (9 drops) and one part of 50 per cent chick embryo extract (3 drops) were freshly mixed and allowed to clot in a watch-glass. The BSS used for preparing the embryo extract contained 1 per cent glucose instead of 0.1 per cent. Fifty units each of penicillin and streptomycin were added to each milliliter of embryo extract.

Vitamin A (crystalline acetate)[1] was added to the basic medium by incorporating the alcoholic solution of vitamin A directly into the plasma (10 to 40 mg vitamin A per ml of absolute ethanol added 0.1 per cent). Final concentration of added vitamin A was 10 to 40 $\mu$g per ml of medium. This is defined as the vitamin A (A+) medium. The normal medium (A−) received the same amount of ethanol minus the vitamin.

*Viruses.*—The influenza A (PR8) virus was originally obtained from Dr. R. E. Shope.

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[1] Vitamin A acetate crystalline, Distillation Products, Division of Eastman Kodak Co., Rochester, New York.

Infectivity titer of the seed virus was $10^{7-8}$ $EID_{50}$/0.1 ml; it has been maintained in our laboratory by routine allantoic passage in chick embryos for 15 years, or about 20 passages. The vaccinia virus, obtained from Dr. R. R. Wagner of the Johns Hopkins Medical School, had an original titer of $2 \times 10^6$ pock-forming units (PFU)/0.1 ml.

*Virus Inoculation of Organ Cultures.*—Cultures which had been maintained in both (A+) and (A−) media for designated times were simultaneously inoculated with virus. The virus inoculum (0.05 ml per culture) was spread over the surface of the tissue explants. After an adsorption period of 1 hour (influenza) or 3 hours (vaccinia) at 36°C, infected cultures were washed in BSS, transferred to fresh medium, and reincubated at 36°C.

*Virus Titration.*—

*Influenza virus:* Each individual pool of 4 to 6 tissue explants was homogenized in a Tenbroeck tissue grinder in 1 ml cold phosphate-buffered saline, then centrifuged at 2500 RPM for 3 minutes. Ten-fold dilutions were made from the supernatant and 0.1 ml aliquots injected into the allantoic sac of 10 to 11-day chick embryos, 4 to 6 eggs per dilution. Allantoic fluids from inoculated eggs were individually tested for hemagglutinin after 48 hours at 36°C. The $EID_{50}$ was calculated by the method of Reed and Muench.

*Vaccinia virus:* Tissue specimens were homogenized as in the influenza virus titration except that the 10-fold dilutions were made in normal saline, and 0.1 ml aliquots inoculated on to the chorio-allantoic membrane of 12-day chick embryos, 4 eggs per dilution. Pocks were counted after 64 hours at 36°C, and virus titer in PFU calculated by multiplying the average count by the dilution factor.

*Histological Procedures.*—Specimens of tissue explants on rayon strips were fixed in Zenker's (3 per cent acetic acid) or Bouin's solution for 30 minutes. If fixed in Zenker's they were subsequently washed in tap water for several hours. Dehydration from 30 to 100 per cent ethanol was carried out at ½ hour intervals. From absolute ethanol the tissues were transferred to 2 changes of acetone for 1½ to 2 hours with frequent shaking to dissolve the rayon fabric, followed by 2 changes of toluene, ½ hour each, and finally embedded in paraffin. Four to 6 $\mu$ paraffin sections were stained as a routine with hematoxylin and eosin. Treatment with 0.5 per cent iodine and 0.75 per cent thiosulfate were included after Zenker's fixative.

*Preparation of Influenza Virus Antiserum.*—Domestic rabbits of 3 to 4 kg were each injected intravenously with 5 ml of undiluted allantoic fluid containing influenza A (PR8) virus, and 1 week later were given 10 ml of the same inoculum intraperitoneally. Serum samples were collected 7 to 10 days after the last injection. Those having a hemagglutination inhibition titer of 1280 or higher against 4 units of influenza virus were used for fluorescein conjugation.

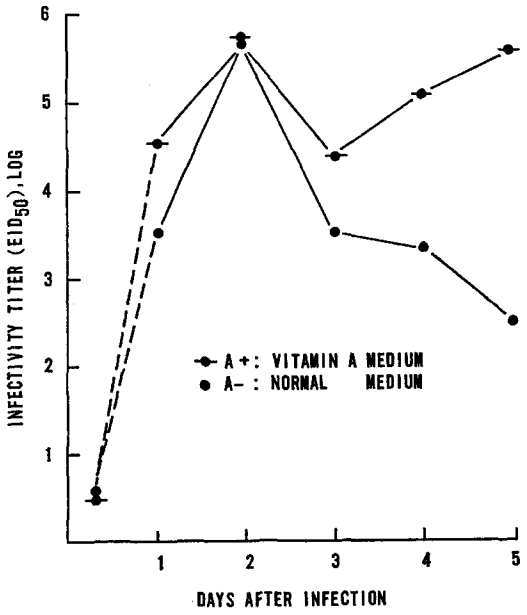
## EXPERIMENTAL RESULTS

*Squamous Differentiation and Vitamin A–Induced Mucous Metaplasia in Organ Cultures of Chick Embryonic Epidermis.*—The uncultured epidermis of 12 day chick embryonic metatarsus skin usually consisted of a single layer of basal cells, two or three intermediate layers, and one or two layers of flat cells on the surface.

After 2 to 3 days' cultivation in organ culture with normal medium (A−) one or two additional intermediate layers of polyhedral cells were produced in the epidermis (Fig. 1). Stratified squamous epithelium usually developed after 4 to 5 days. Early keratinization began at the 6th or 7th day of cultivation, and eosinophilic granules of keratohyalin and pyknotic nuclei appeared in the sur-

face cells (Fig. 2). On further cultivation, layers of cornified elements were added to the epidermal surface. Formation of scales became evident after 6 to 7 days in normal medium.

In the vitamin A medium (A+), (40 μg/ml) organ cultures of epidermis showed no appreciable difference from those in (A−) medium during the first 2 to 3 days. After 4 to 5 days in (A+) medium, the superficial layers of flat cells became detached, and the underlying polyhedral cells showed morphological



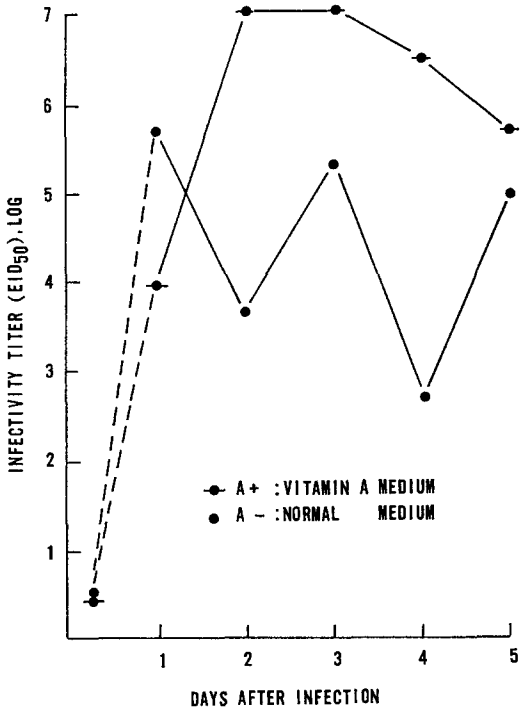

TEXT-FIG. 1. Influence of excess vitamin A (40 μg/ml) on the growth of influenza (PR8) virus in organ cultures of 12 day chick embryonic epidermis infected at the time of explantation.

changes and rearrangement. By about the 6th or 7th day, a new surface epithelium made up of cuboidal or columnar cells usually developed. A scattering of mucous cells in this surface layer increased in number when the tissue was transferred from the vitamin A (A+) to the normal medium (A−) at about this period. In contrast to tissues which were constantly maintained in (A−) medium, squamous keratinization and scale formation were both inhibited in tissues grown in (A+) medium. These results concur with those first reported by Fell and Mellanby (5) and Fell (6) (Figs. 1 to 11).

*Susceptibility of the Epidermis to Influenza Virus at the Time of Explantation with and without Excess Vitamin A.*—Since the degree and type of differentiation of the epidermis in organ culture were related to the period of cultivation in

the presence or absence of excess vitamin A, influenza virus was then inoculated at different times of treatment: immediately on explantation and after 4 and 8 days of cultivation in normal or vitamin A medium, representing respectively, predifferentiation, mid-stage and late stage of squamous keratinization or mucous metaplasia.

The data on susceptibility at the time of explantation will be presented first.



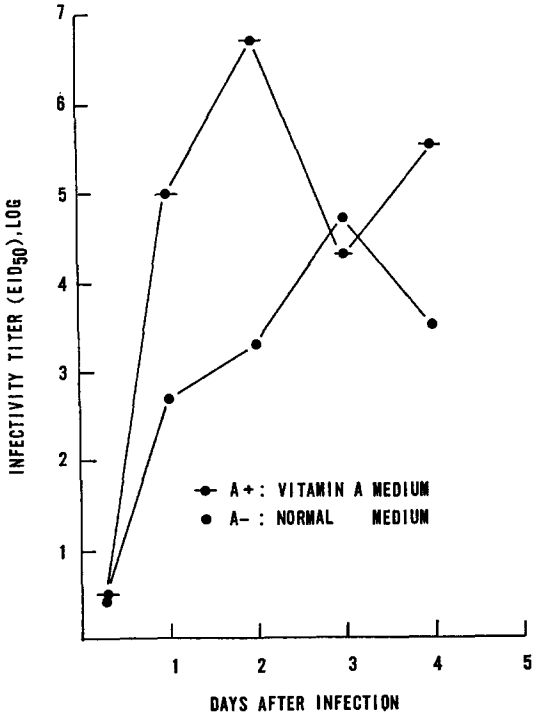

TEXT-FIG. 2. Influence of 4 days' cultivation with excess vitamin A (40 $\mu$g/ml) on the susceptibility of 12 day chick embryonic epidermis to influenza virus in organ culture.

In both the (A−) and (A+) groups, a peak titer of $10^{5.7}$ $EID_{50}$ was attained after 2 days of infection. Virus production then fell at 3 and 5 days in the (A−) cultures, while a smaller second rise of virus production was obtained in the (A+) cultures, producing a difference of 50- to 1000-fold after 4 to 5 days of infection.

Thus the maximum yield of virus did not differ in the tissues grown in the two types of media during the early stage of infection, but significantly more virus was later produced in the (A+) medium (Text-Fig. 1).

*Susceptibility of the Epidermis to Influenza Virus after 4 Days in Organ Culture with and without Excess Vitamin A.*—Epidermis grown for 4 days in organ cul-

ture and then inoculated with virus showed a marked difference in virus production dependent upon the presence or absence of excess Vitamin A. A maximum infectivity titer of $10^{5.7}$ $EID_{50}$ was attained by the (A−) cultures 1 day after infection; subsequent titers in this series fluctuated considerably, but none exceeded this yield.

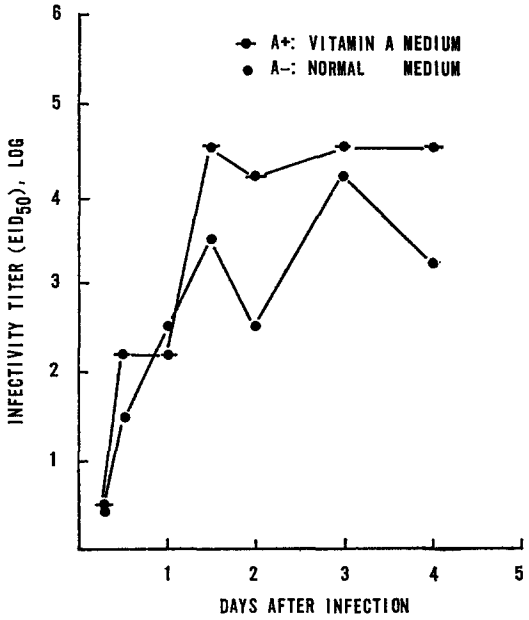



TEXT-FIG. 3. Influence of 8 days' cultivation with excess vitamin A (40 μg/ml) in organ culture on the susceptibility of 12 day chick embryonic epidermis to influenza virus.

In comparison, the (A+) cultures (40 μg/ml) reached a peak titer of $10^{7.0}$ $EID_{50}$, by 2 days or 20-fold over that of the (A−) group at the peak. The excess in virus yield was 5- to 6300-fold ($10^{0.7-3.8}$ $EID_{50}$) in the (A+) cultures for the period of 5 days of infection (Text-fig. 2).

*Susceptibility of the Epidermis to Influenza Virus after 8 Days in Organ Culture with and without Excess Vitamin A.*—The highest yield of influenza virus attained by the normal medium (A−) group of cultures infected on the 8th day was $10^{4.7}$ $EID_{50}$, which is 10-fold lower than the peak titer of the 4- and the 0-day-infected (A−) groups. Thus the maximum yield of influenza virus in the epidermis organ cultures kept for 8 days without excess vitamin A is much less than that of untreated freshly cultured skin (Text-figs. 1 to 3).

The vitamin A (A+) cultures infected on 8th day showed a peak virus yield of $10^{6.7}$ $EID_{50}$ 2 days after infection (Text-fig. 3), which is 10-fold higher than the peak titer of both (A−) and (A+) groups infected on 0 day (Text-fig. 1). Influenza virus growth thus was not enhanced by vitamin A when infection was initiated at the time of explantation, but may have been enhanced by growth in the presence of excess vitamin A for 4 or 8 days before inoculation (Text-fig. 3).




TEXT-FIG. 4. Change of susceptibility in organ culture of 10 day chick embryonic epidermis after 8 days' cultivation with low dose (13 μg vitamin A per ml plasma) of added vitamin A.

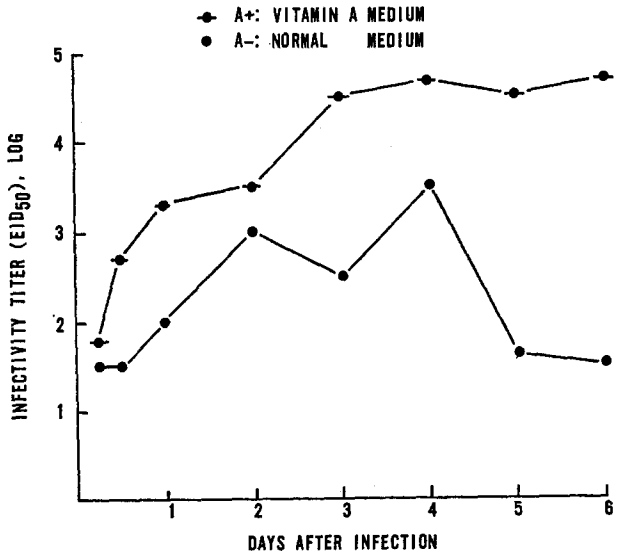
These results may be summarized as follows:

(*a*) In the predifferentiation period, the maximum yield of influenza virus did not differ between the normal and vitamin A media.

(*b*) After 4 days of cultivation in each type of medium, the relative susceptibility of the epidermis was altered. The maximum yield of influenza virus in the (A+) cultures was increased 20-fold over its yield at predifferentiation period. The yield was unchanged in the (A−) group. Thus a difference of 5- to 6300-fold in range was produced, and was maintained for 5 days.

(*c*) After 8 days of cultivation, susceptibility to influenza virus was still greater than at its predifferentiation level in the (A+) group but had remained steady or somewhat decreased in the (A−) cultures. The difference in the maximum virus yield between the two medium groups was thus maintained.

*The Effect of Lower Doses of Vitamin A Maintained for Longer Periods of Time.*—The experimental conditions were altered somewhat for this comparative study. Younger (10 day) chick embryonic skin from trunk and limbs, rather than from metatarsus, was infected after prolonged cultivation periods (8 to 11 days). Lower concentrations of vitamin A (13 $\mu$g/ml plasma) and larger inocula of influenza virus ($0.5 \times 10^6$ $EID_{50}$ per culture) were used. Infected cultures were then incubated without washing, and media were not renewed.

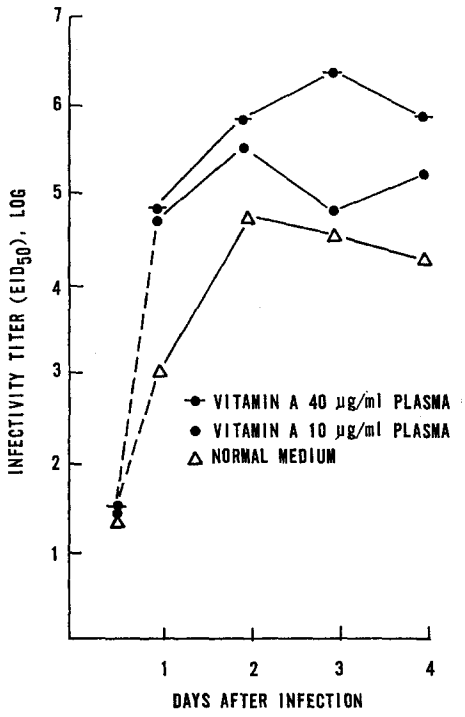



TEXT-FIG. 5. Change of susceptibility to influenza virus in organ culture of 10 day chick embryonic epidermis after 11 days' cultivation with low dose (13 $\mu$g) of added vitamin A.

A higher yield of influenza virus was obtained from the (A+) cultures than from the (A−) when the epidermis was infected after 8 days' or 11 days' cultivation. The difference between the two groups was consistent over the entire 4 to 6 day observation period. A larger difference was found in the cultures grown for 11 days before infection. Virus titer declined in the (A−) group, but remained high in the (A+) group (Text-figs. 4 and 5).

*Relation to Vitamin A Concentration.*—Three sets of organ cultures of metatarsal epidermis of 12-day chick embryos were grown for 4 days or 8 days; two were maintained in differing concentrations of vitamin A (10 $\mu$g and 40 $\mu$g per ml plasma), and the third in normal medium, before inoculation with influenza virus. A difference in virus production which correlated with the concentration of added vitamin A was apparent in cultures infected both after

4 and 8 days' cultivation. The difference of virus yield was small between the normal medium and the low vitamin A group (the latter induced only partial mucous metaplasia in the epidermis in 4 to 8 days). The difference between the normal medium and the high vitamin A group was significant. The over-




TEXT-FIG. 6. Effect of 4 days' cultivation with different concentrations of added vitamin A on the change of susceptibility to influenza virus in organ culture of 12 day chick embryonic epidermis.

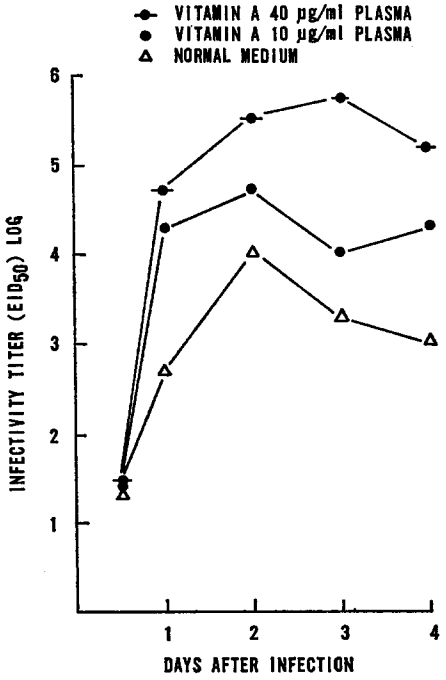
all proportional difference in virus titer among the three groups of cultures was clear and consistent (Text-figs. 6 and 7).

Thus the magnitude of increase in influenza virus production in this organ culture system is directly related to the amount of excess vitamin A present in the culture medium 4 and 8 days before the inoculation of virus. These results suggest a specific role of vitamin A in the change in susceptibility of the epidermis.

*Effect of Vitamin A on Growth of Influenza Virus in Tissue Cultures of Chorio-Allantoic Membrane.*—The important question of whether vitamin A itself exerts any effect on influenza virus growth remained.

In this experiment two concentrations of vitamin A were added to roller tube cultures of chorio-allantoic membrane from 11-day chick embryos. Each culture contained approximately 25 pieces of 2 to 3 mm explants in 3 ml of Hanks' balanced salt solution. Four kinds of media were used: (*a*) vitamin A 40 μg/ml medium and 0.1 per cent of ethanol; (*b*) vitamin A 10 μg/ml medium and 0.1 per cent of ethanol; (*c*) ethanol control, 0.1 per cent ethanol without vitamin A; (*d*) saline control, saline 0.1 per cent (0.1/100 medium).

Influenza virus ($0.5 \times 10^6$ $EID_{50}$ per culture) was inoculated at the beginning of cultiva-



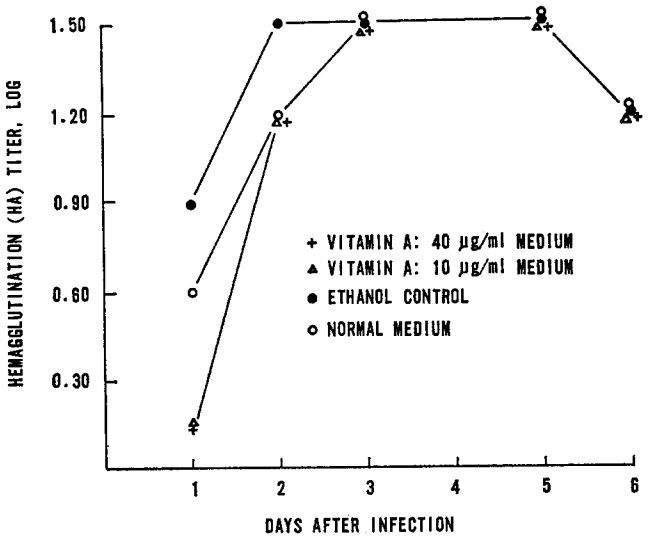

TEXT-FIG. 7. Effect of 8 days' cultivation with different concentrations of added vitamin A on the change of susceptibility to influenza virus in organ culture of 12 day chick embryonic epidermis.

tion. Virus yield was measured as hemagglutinin titer from daily pools of 0.3 ml of culture fluids, which were replaced by 0.3 ml of fresh medium.

There was practically no difference in the maximum yield of influenza virus among the 4 groups of cultures 1 to 4 days after infection. After some variation in the initial rate of increase of titer the subsequent peak titer was the same in all 4 groups 2 to 3 days after infection, and decreased in all 4 groups on the 6th day. Direct contact with excess vitamin A throughout the course of infection thus had no detectable effect on influenza virus or its propagation in tissue cultures of chorio-allantoic membrane (Text-fig. 8). Since increasing doses of added vitamin A had no immediate effect on influenza virus growth, the greater

yield of this virus in tissues grown for 4 or more days in organ culture with excess vitamin A before inoculation of virus probably is not due to the continual presence of the vitamin itself. Its apparent dependence on a period of precultivation implies that the specific effect of vitamin A is mediated through a sequence of changes occurring in the organized tissues of the explants (Fig. 9).

*The Initial and Subsequent Growth of Influenza Virus in Organ Cultures of Chick Embryonic Epidermis.*—To obtain information on the early phase of influenza virus growth in the organ culture system, 0- and 4-day cultures of epidermis in (A−) and (A+) media were inoculated with two different amounts



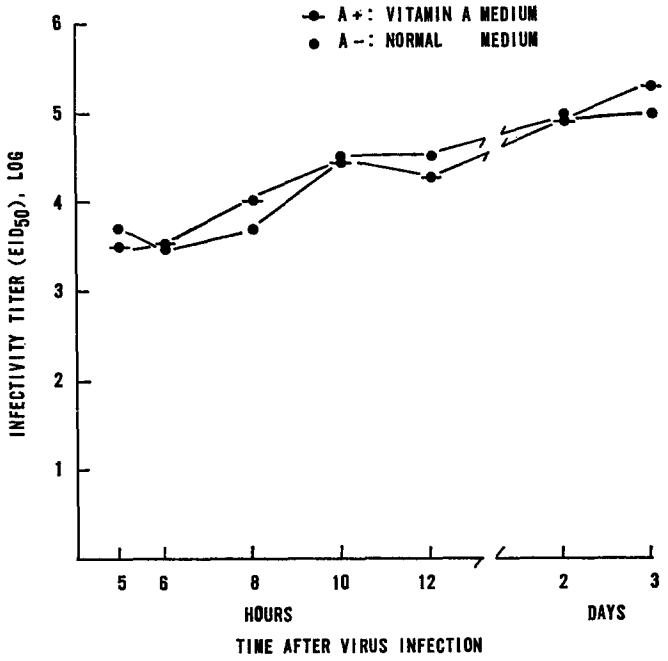

TEXT-FIG. 8. Lack of effect of vitamin A on the growth of influenza virus in tissue culture of chorio-allantoic membrane.

of influenza virus ($0.5 \times 10^2$ and $0.5 \times 10^7$ $EID_{50}$) per culture. In a second experiment, the virus yield following 2 to 3 days of infection was also measured. No significant difference in early virus production (2 to 12 hours) was found between the (A−) and (A+) groups of cultures (Text-fig. 9). There was, however, a higher yield in the (A+) group at 2 to 3 days, but the increase of virus was observed only in cultures in which the infection had been initiated 4 days after explantation (Text-fig. 10). A difference in size of virus inoculum had little effect on the results. (Text-figs. 11 and 12).

*The Growth of Vaccinia Virus in Organ Cultures of Chick Embryonic Epidermis.*—In order to define the specificity as well as the possible mechanism involved in the change of susceptibility of embryonic skin organ cultures, the comparative growth of a dermotropic virus (vaccinia) was studied. Organ

cultures were prepared from 12 day chick embryonic metatarsal epidermis. At specified periods of cultivation, ($0.7 \times 10^4$ PFU per culture) vaccinia virus was inoculated simultaneously onto cultures maintained in (A−) and (A+) media, and the subsequent daily yield of virus was determined.

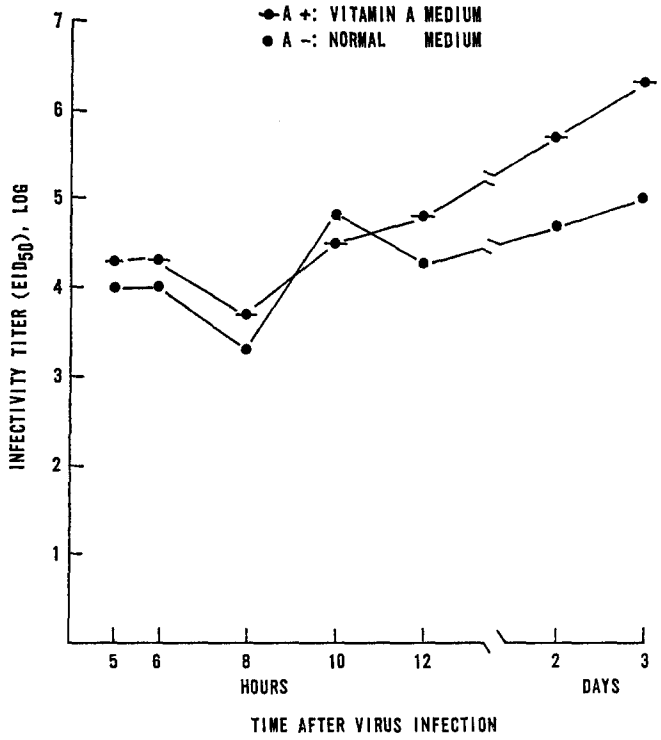
The yield of virus from cultures infected at the times of explantation showed no significant difference when grown on (A−) and (A+) media. The virus titer



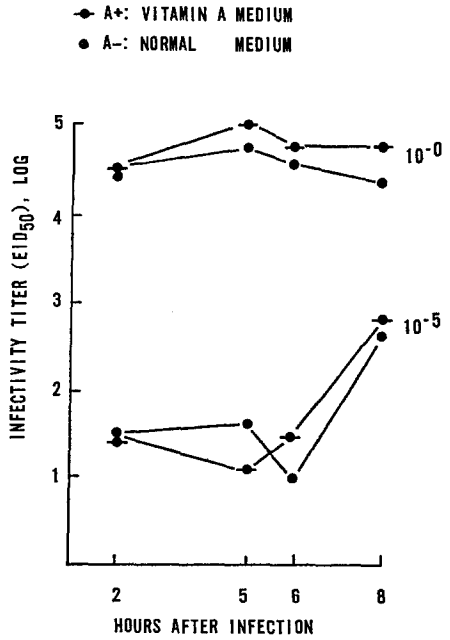

TEXT-FIG. 9. Lack of effect of excess vitamin A on the initial and subsequent rises of influenza virus titer in organ cultures of 12 day chick embryonic epidermis infected at the time of explantation

rose in both groups from 1 to 4 days, when it reached a peak titer of 44 to $45 \times 10^4$ PFU, then declined in both groups after 5 days.

Four days' cultivation in organ culture before inoculation of vaccinia virus caused a significantly lower yield of this virus in the vitamin A (A+) group. This difference was not observed until 3 to 4 days after infection, when the virus titer began to fall in the (A+) cultures, yet continued to rise in the (A−) group. Prolonging the cultivation period to 8 days before infection produced essentially the same growth pattern as the infection initiated on day 4. The rise of virus titer was closely similar in both (A−) and (A+) cultures from 1 to 3 days, but at 4 days the yield was significantly higher in the (A−) group (Text-Figs. 13 to 15).

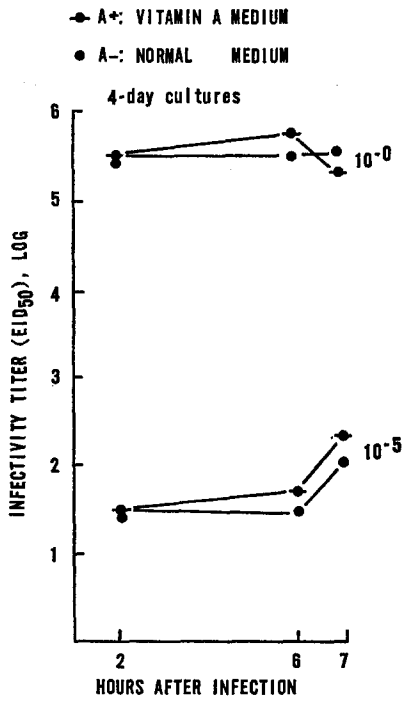



TEXT-FIG. 10. Influence of excess vitamin A on the initial and subsequent rises of influenza virus titer in organ cultures of 12 day chick embryonic epidermis infected 4 days after explantation.



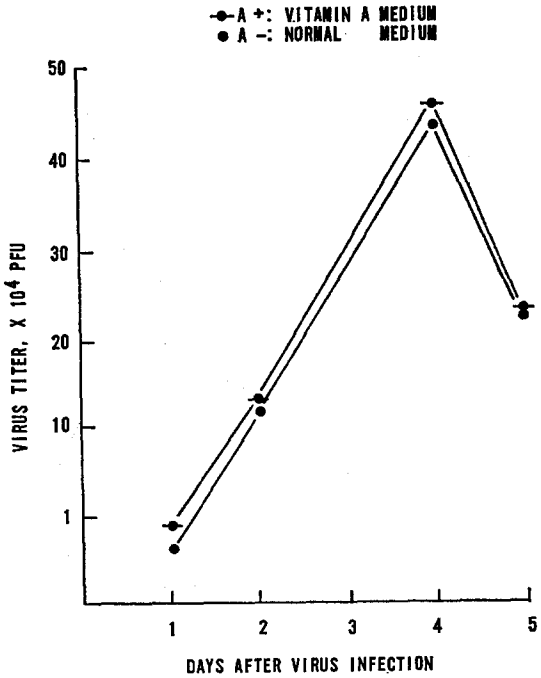

TEXT-FIG. 11. Influence of size of inoculum on the initial rise of influenza virus titer in organ culture of 12 day chick embryonic epidermis.

Cultures infected with $10^{-0}$ and $10^{-5}$ dilutions of influenza (PR8) virus at the beginning of cultivation in normal and vitamin A media.

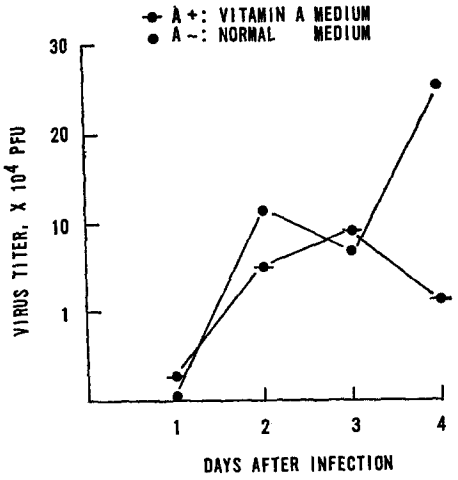



TEXT-FIG. 12. Influence of size of inoculum on the initial rise of influenza virus titer in organ culture of 12 day chick embryonic epidermis.

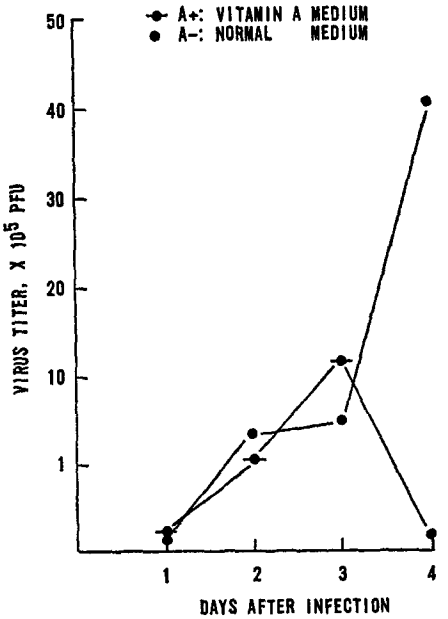
Cultures infected with $10^{-0}$ and $10^{-5}$ dilutions of influenza (PR8) virus after cultivation for 4 days in vitamin A and normal media.




TEXT-FIG. 13. Influence of excess vitamin A on the growth of vaccinia virus in organ cultures of 12 day chick embryonic epidermis infected at the time of explantation.




TEXT-FIG. 14. Influence of 4 days' cultivation with excess vitamin A on the susceptibility of 12 day chick embryonic epidermis to vaccinia virus in organ culture.




TEXT-FIG. 15. Influence of 8 days' cultivation with excess vitamin A on the susceptibility of 12 day chick embryonic epidermis to vaccinia virus in organ culture.

## DISCUSSION

Organ culture offers a convenient tool for analysis of tissue differentiation *in vitro*. Such characteristics were correlated with tissue specificity to virus infection in the present study. Fell and Mellanby (5) have reported that the epidermis of chick embryos undergoes squamous keratinization in organ culture, but converts to mucous epithelium in response to excess vitamin A. Using this model, the special affinity of influenza virus to mucous epithelium was examined in relation to such metaplasia *in vitro*.

The results suggest that a changing pattern of *in vitro* differentiation is accompanied by a corresponding change of virus specificity. The PR8 strain of influenza virus, although able to grow in the skin of young chick embryos, grew far better when the differentiation of the epidermis was diverted from normal squamous to mucous epithelium. On the other hand, mucous differentiation gave no advantage to the growth of vaccinia virus which is adapted to epidermal epithelium. Differentiation, although generally lost in cell cultures, can be closely reproduced in organ culture. The following factors may be involved in the change of virus yield:

1. Barrier of cornified tissue: squamous keratinization starts in the skin organ culture about the 4th day of cultivation in normal (A−) medium. The cornified layers of inert keratin fibers may bar the infecting virus particles from the viable cells underneath. Early yield (2 to 12 hours) of influenza virus in this organ culture system, however, shows no significant difference between (A−) and (A+) groups. A clear difference was usually shown only 2 to 3 days following infection, coincident with the period of active virus increase, or peak infectivity titer. This suggests that the differential factors involved are probably built up through a series of virus cycles, rather than at the initiation of the infection. Furthermore the greater yield of vaccinia virus from the keratinized cells also argues against a barrier effect or lack of viable cells in the keratinized tissue. The latter is reminiscent of the growth of rabbit papilloma virus which has special preference for keratinized tissue (9, 10).

2. Antikeratinizing effect of vitamin A: since the increase of influenza virus in the (A+) cultures not only compensated for the decrease of virus yield in the (A−) group at corresponding cultivation periods, but exceeded its predifferentiation level of virus production, the better yield of influenza virus in the (A+) cultures can not be solely attributed to an antikeratinizing effect of vitamin A.

3. Relative number of cells available: the flattened squamous epithelial cells which developed in the epidermal surface after 4 days in the normal (A−) medium are approximately 2 to 3 times wider in their cross-section than the surface cuboidal epithelial cells in the (A+) cultures at a corresponding period. If due allowance is made for the elevated areas of scales formed in the (A−) but not in the (A+) cultures which perhaps increase total surface area

by 4- to 5-fold, the net difference is small. Direct count of surface cells in stained sections shows approximately 2 times more cells in cross-section, or 4 times more per square area in the (A+) cultures. Experiments on the early (2 to 8 hours) yield of influenza virus revealed no significant difference between (A+) and (A−) groups. Continual rise of virus titer then occurred 1 to 4 days after infection, and a higher yield of virus was eventually associated with the (A+) group. Fluorescent antibody staining showed only about 20 per cent of the surface area involved at 72 hours after infection of influenza virus. Afterwards, the staining area enlarged progressively in both groups of cultures. This suggests that a wide margin of cell surplus existed in both groups at the time when the virus yield of both the (A+) and the (A−) groups was already at maximum. In other words, the relative number of available cells probably is not the factor which lowers the virus yield in the (A−) cultures.

The mechanisms underlying metaplasia and the accompanying change in susceptibility are not known. Cellular differentiation almost certainly involves the establishment of new balances in the enzyme systems. The change in the metabolic pattern of the host cell may selectively affect the attachment or growth of different viruses. Some metabolic effects of vitamin A have been studied by autoradiography (11). In organ cultures of chick embryonic epidermis, vitamin A stimulates the uptake of $S^{35}$-sulfate in the converted mucous epithelium. The reverse of this occurs in squamous keratinized epithelium.

In bone tissue, vitamin A decreases cartilage formation and decreases rather than increases $S^{35}$-sulfate uptake. It causes dissolution of the cartilage matrix, liberation of sulphated mucopolysaccharides, and subsequent loss of metachromasia (12). Comparable effects were observed with papain on young bone tissue *in vivo* (13), and in organ culture (14). It has been proposed that bound protease is released from tissues by vitamin A. A similar proteolytic effect has been found upon exposing normal cartilage tissue to distilled water, and certain proteases are known to be liberated from the intracellular organelle lysosomes under hypoosmotic conditions. Dingle (16) found that vitamin A indeed causes the release of a bound protease from isolated lysosomes of rat liver cells *in vitro*.

Vitamin A appears to play a specific role in the biosynthesis of mucopolysaccharides in the tissue (17). Mucoprotein serves as a receptor for the attachment of influenza virus as well as an inhibitor of hemagglutination. Influenza virus infection is associated with a decrease of mucoprotein content in the upper respiratory tract (18). A decrease of the hemagglutination inhibitor (receptor) titer has been correlated with rise of the hemagglutinin in infected chorioallantoic membrane (19, 20). Schlesinger and Karr (20) have suggested the possible role of mucoprotein as an intracellular substrate for influenza virus growth. Wolff (21) has subsequently shown a constant decrease of glycoprotein

in mouse lung epithelial sections starting 1 to 6 hours after infection with influenza virus.

Vitamin A and several other surface active agents cause the production of irregular filamentous-like forms of influenza virus in the infected chorio-allantoic membrane (22). Since the filamentous form is predominant in newly isolated influenza virus strains in the allantoic sac a similar surface effect on mucous cells should be investigated.

## SUMMARY

The conversion of chick embryonic epidermis to mucous epithelium by excess vitamin A in organ culture as reported by Fell and Mellanby (5) was shown to be accompanied by a corresponding change of susceptibility to influenza and vaccinia viruses.

Untreated epidermis of 10- to 12-day chick embryos supported the growth of influenza (PR8) virus in organ cultures and a maximum infectivity ($EID_{50}$) titer was reached 2 to 3 days after infection. At the same time, the epidermis showed squamous keratinization, beginning about the 4th day of cultivation.

Addition of excess vitamin A (40 $\mu$g per ml) to the skin organ culture induced the following changes: (*a*) mucous metaplasia of the epidermis which was usually first evident after 4 to 5 days in the vitamin A medium, (*b*) increase in the daily and maximum yield of influenza virus, if the epidermis had been grown for 4 or more days in the vitamin A medium before infection took place, and (*c*) decrease in the production of vaccinia virus under similar conditions. The maximum yield of both viruses remained unchanged, however, if excess vitamin A was introduced to the organ culture at the time of virus inoculation.

The magnitude of increase in the yield of influenza virus in this organ culture system was found to be proportionally related to the concentration of vitamin A added 4 or more days before inoculation of this virus. Increasing doses of vitamin A however, had no effect on the short-term growth of influenza virus in tissue cultures of chorio-allantoic membrane.

Observation on the early period (2 to 12 hours) of influenza virus growth initiated in the 4-day organ cultures of chick embryonic skin showed no significant difference in virus production between the normal and the vitamin A medium groups.

The change of virus specificity apparently is not due to the presence of excess vitamin A *per se*, but appears to be related to the change of differentiation produced in the organ culture system.

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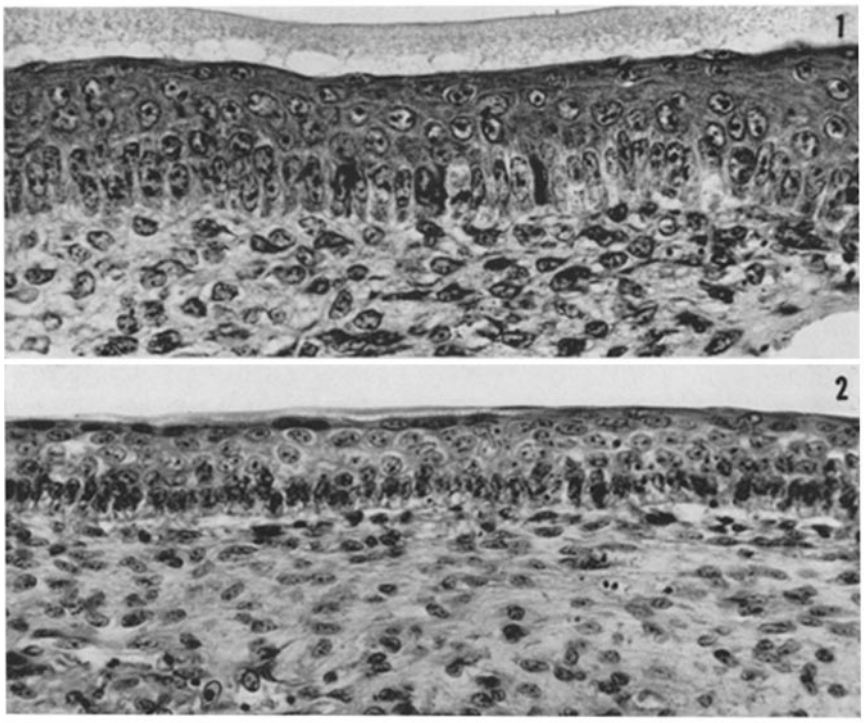
## EXPLANATION OF PLATES

### PLATE 9

FIG. 1. Explant of 12 day chick embryonic skin (shank) after cultivation for 1 day in organ culture with vitamin A medium (40 $\mu$g vitamin A added per ml plasma). The epidermis consists of a basal layer, three intermediate and one to two superficial flattened strata. Hematoxylin and eosin. × 400.

FIG. 2. Explant of 12 day chick embryonic skin (shank) after cultivation for one day as organ culture with normal medium. Hematoxylin and eosin. × 400.

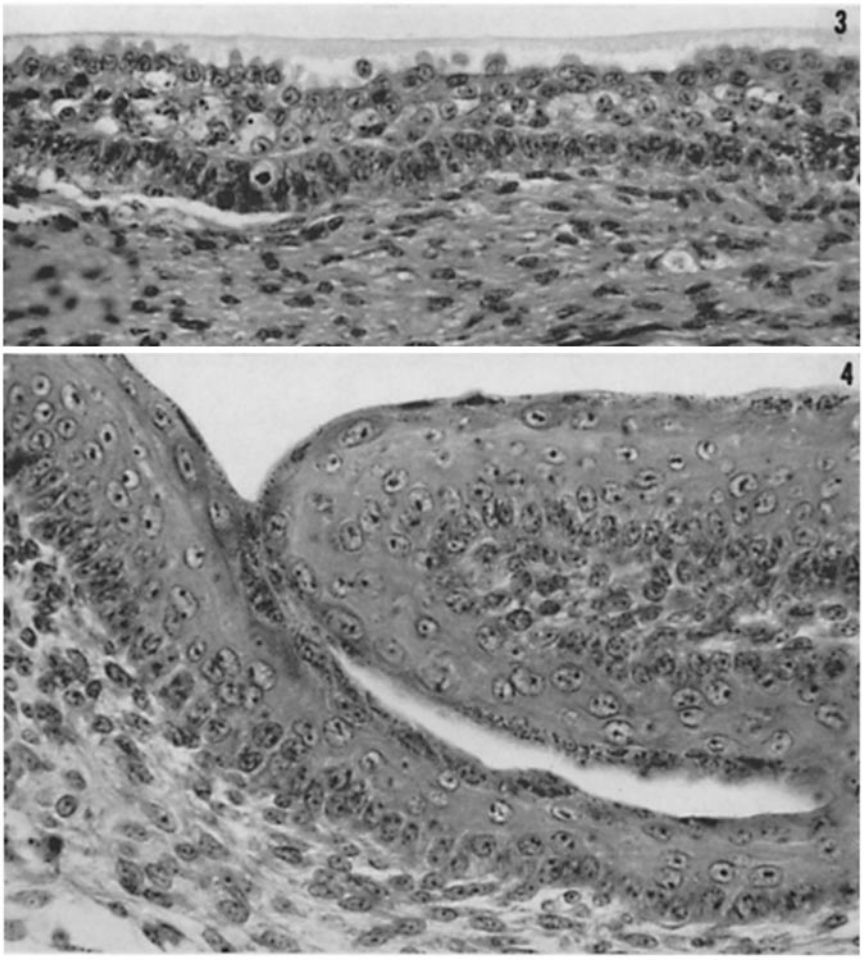



(Huang and Bang: Influenza virus following excess vitamin A)

PLATE 10

FIG. 3. Four day organ culture of 12 day chick embryonic skin (shank) in vitamin A medium. The superficial layers of flattened cells are already detached. Reorganization of the epidermis is in process. Hematoxylin and eosin. × 400.

FIG. 4. Four day organ culture of 12 day chick embryonic skin (shank) in normal medium, showing early squamous keratinization, scale formation, and the presence of eosinophilic keratohyalin granules in the surface cells. Hematoxylin and eosin. × 600.

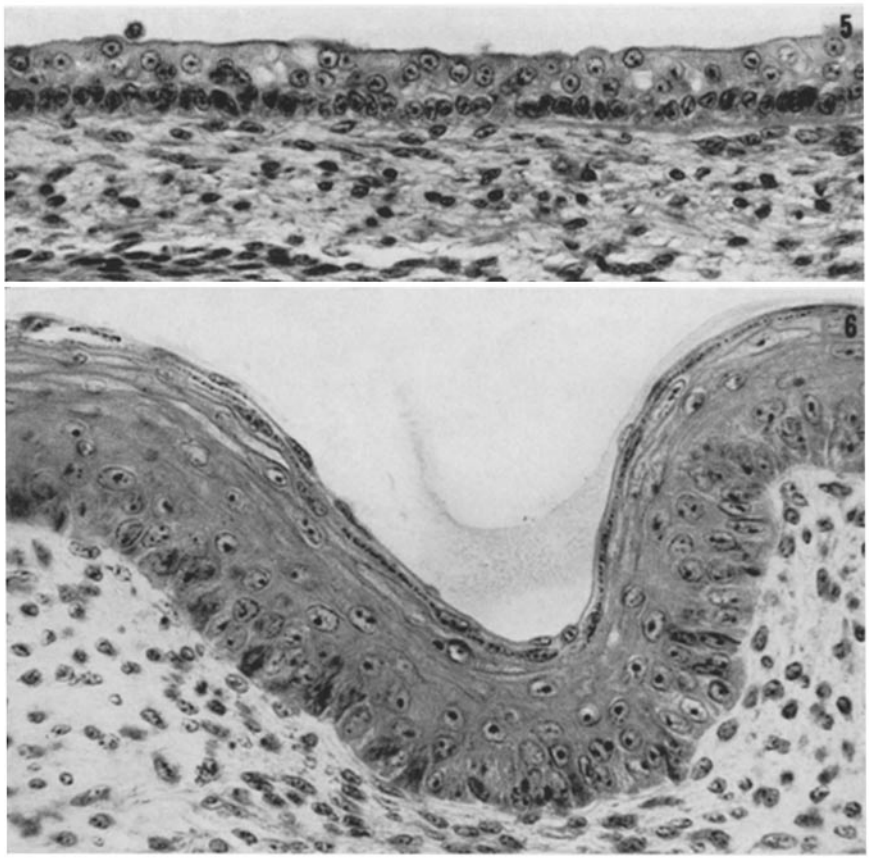



(Huang and Bang: Influenza virus following excess vitamin A)

PLATE 11

FIG. 5. Five day organ culture of 12 day chick embryonic skin (shank) in vitamin A medium. Hematoxylin and eosin. × 400.

FIG. 6. Five day organ culture of 12 day chick embryonic skin (shank) in normal medium. Hematoxylin and eosin. × 600.

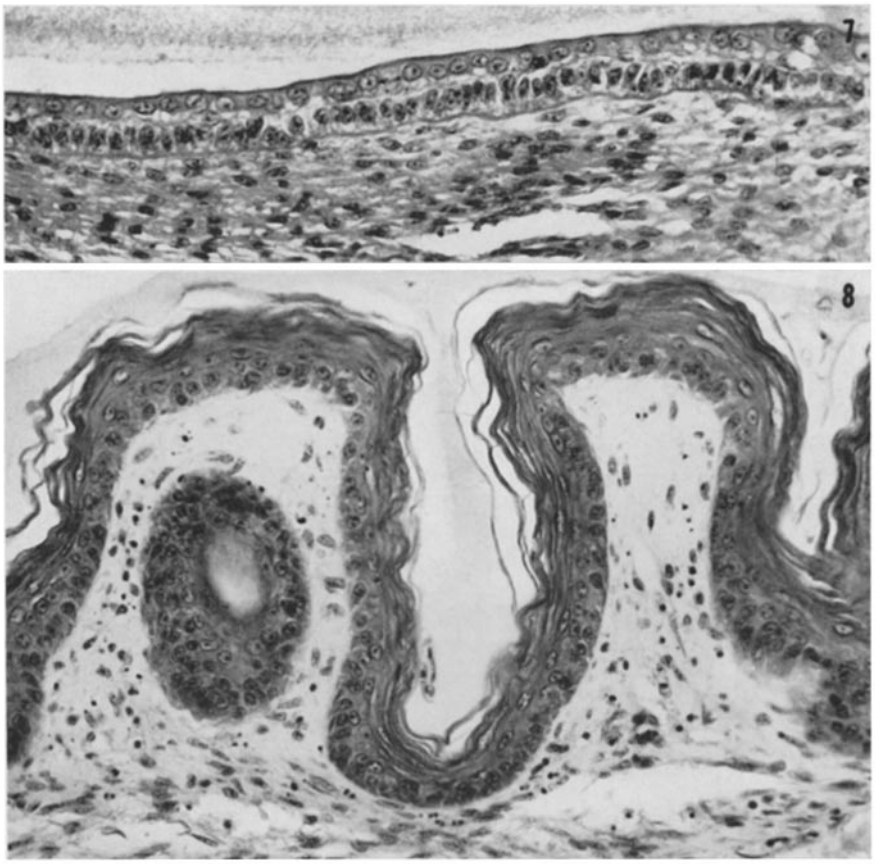



(Huang and Bang: Influenza virus following excess vitamin A)

PLATE 12

FIG. 7. Eight day organ culture of 12 day chick embryonic skin (shank) in vitamin A medium. The epidermal surface is lined with cuboidal epithelium. Hematoxylin and eosin. × 400.

FIG. 8. Eight day organ culture of 12 day chick embryonic skin (shank) in normal medium. Advanced stage of keratinization. Hematoxylin and eosin. × 600.



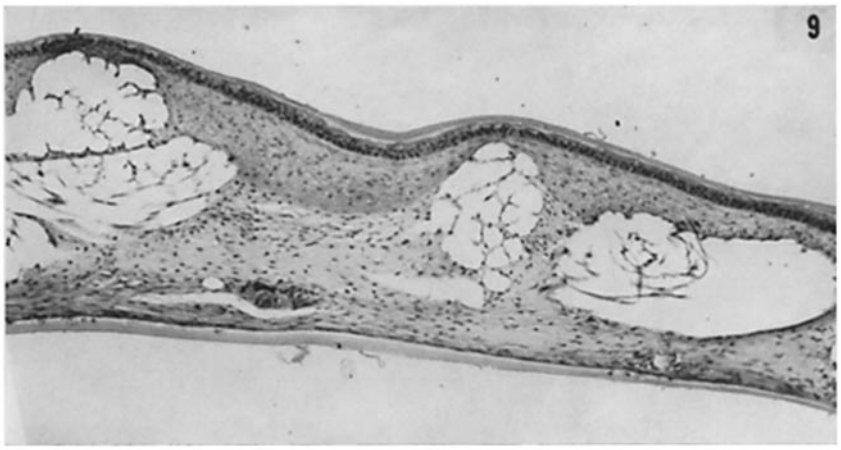

(Huang and Bang: Influenza virus following excess vitamin A)

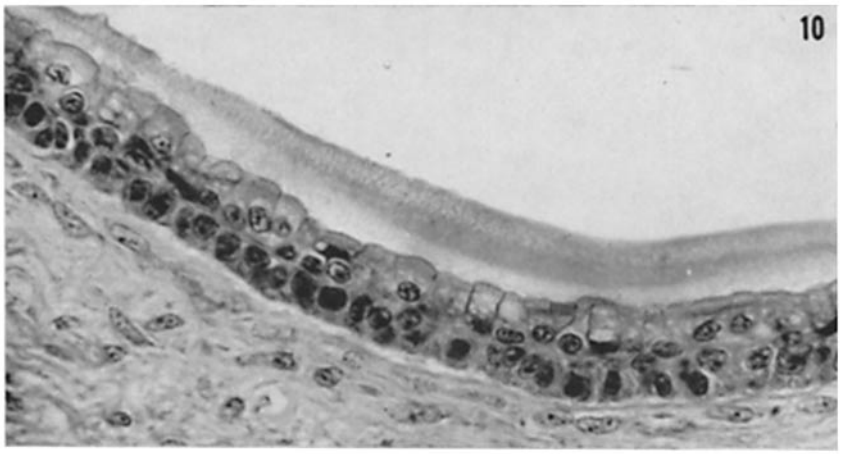
PLATE 13

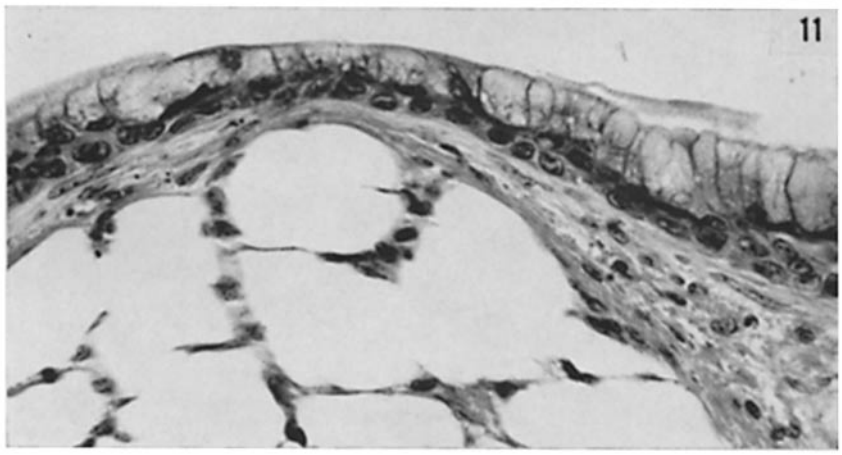
FIG. 9. Explant of 12 day chick embryonic skin (shank) after 6 days' cultivation in organ culture with vitamin medium followed by 3 days with normal medium. The empty spaces at the central portion of the section are the "ghosts" of the rayon acetate fibers which have been dissolved out with acetone. Hematoxylin and eosin. × 100.

FIG. 10. Same section as Fig. 9. Note the goblet cells developed amid the cuboidal and columnar epithelial cells on the surface of the epidermis. Hematoxylin and eosin. × 600.

FIG. 11. Explant of 12 day chick embryonic skin (shank) after 7 days' cultivation in organ culture with vitamin A medium followed by 3 days with normal medium, showing typical goblet cells lining the surface. Hematoxylin and eosin. × 600.




(Huang and Bang: Influenza virus following excess vitamin A)