MULTIPLE VIRUS INFECTION OF SINGLE HOST CELLS*

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PLATES 15 AND 16

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In considering the intracellular parasitism that characterizes the host-virus relationship, it has often been tacitly assumed that the parasitization of an individual host cell is limited to infection by a single virus. In contradiction to this belief, however, we have reported experiments (1) in which the cells of a virus-induced tumor, Shope's rabbit papilloma (2), and of a carcinoma of the sort that frequently succeeds the benign growths (3, 4), were superinfected by other viruses—"superinfected" in the case of the papilloma because its cells contained the causative virus. These same studies, furthermore, included certain instances in which the simultaneous introduction of two viruses for the purpose of inducing superinfection was followed by evidence of the simultaneous activity of both of the viruses within individual cells. This evidence was the presence of two diagnostically significant inclusion bodies, the one cytoplasmic, the other intranuclear, within a single cell. In making our first report, we recognized that the histopathological examination of a stratified epithelial structure, such as the rabbit papilloma, involved the likelihood of error—of the mistaken interpretation that two overlying cells constituted a single cell. If such an error were made, the conclusion might be reached that the two inclusion bodies, cytoplasmic and intranuclear, were present in a single cell, when one of the inclusion bodies might actually be in one host cell and the other inclusion body in an overlying cell. It was for the purpose of eliminating, as far as possible, this opportunity for error that the present investigation was undertaken. The results of these experiments (5), which will now be described in detail, give evidence that an individual host cell can be simultaneously parasitized by more than one virus and amply confirm our earlier findings.

Materials and Methods

The five viruses employed in the present investigations were vaccinia, myxoma virus, B virus, herpes virus, and virus III. The strains of virus employed, and the method for the preparation of the virus suspensions have already been described in detail (1 *c*).

Normal host cells for experimental infection were provided by using rabbit's cornea, skin, or testicle. Of the eleven rabbits employed, nine were domestic stock rabbits (*Oryctolagus*)

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and two were cottontails (*Sylvilagus*). The technique employed for the infection of corneal cells was to scarify both corneas by cross-hatching with a cataract knife, instill 0.2 ml. of virus mixture into the conjuctival sac of the right eye, and gently massage the cornea with the overlying lid. The scarified cornea of the left eye served as the control. The attempted infection of the cells of the skin and testicle was effected by infiltrative inoculation, as previously described (1 *c*).

Tissues for histopathological study were removed immediately after the rabbits had been killed by chloroform anesthesia. Representative blocks were fixed in Zenker's (5 per cent acetic acid) fixative fluid, and embedded in paraffin. The sections were stained with hematoxylin and eosin as routine, and according to Giemsa's method. Exceptionally, these stains were supplemented by phloxin-methylene blue and eosin-methylene blue.

EXPERIMENTAL

In an attempt to establish the infection of a single host cell by more than one virus, five experiments were undertaken. Each was based on the use of a different combination of viruses. The viruses in combination were selected so that one virus would give rise to inclusion bodies in the nucleus, and the other virus to inclusion bodies in the cytoplasm. Examples of such combinations are herpes virus and vaccine virus; B virus and myxoma virus.

Simultaneous Infection of the Normal Cornea with Vaccinia and B Viruses

Mixtures of vaccinia virus and B virus, after being prepared as indicated in the individual protocols, were used for Experiments 1 and 2.

Experiment 1.—Three domestic rabbits were used. The mixture of viruses utilized for instillation into the right conjunctival sac of each rabbit consisted of a 10 per cent suspension of virus-containing tissues. This mixture was prepared by combining 1 part of tissue containing vaccinia virus with 2 parts of tissue containing B virus. The vaccinia virus was derived from chorio-allantoic membranes of chicks. The B virus was obtained from the brain and cord of a moribund rabbit.

Two of the rabbits were killed 48 hours after inoculation. At that time keratitis was present in the right corneas of both rabbits. The left corneas showed negligible evidence of reaction along the lines of scarification.

Rabbit A: Examination of the sections revealed many Guarnieri bodies as evidence of vaccinia virus infection and many intranuclear inclusion bodies of type A as evidence of B virus infection. Both types of inclusion bodies were present in neighboring cells as well as in individual cells (Fig. 1).

Rabbit B: Although there were abundant inclusion bodies, both cytoplasmic and intranuclear, in different cells, in no instance could both a cytoplasmic and intranuclear inclusion body be demonstrated in the same cell.

Rabbit C: This rabbit was killed 72 hours after inoculation. Examination of the sections revealed extensive necrosis of the cornea and abundant evidence of specific virus activity in many of the remaining epithelial cells. Numerous cytoplasmic and intranuclear inclusion bodies were present. One area proved to be particularly worthy of careful study, for it revealed two cells separated from all the adjacent cells (Fig. 2). Within each of these cells a Guarnieri body and an intranuclear inclusion body of type A were found. Serial sections conclusively established the singleness of the cells in question and served further to rule out

the possibility of artefacts. These points were definitely established by photomicrographs taken in four optical planes in black and white (Fig. 3) and at three optical planes in color (Fig. 5).

Experiment 2.—One domestic rabbit, rabbit D, was used. The procedure followed that outlined above, except that the inoculum was composed of equal parts of a 10 per cent brain-cord suspension of B virus and vaccinia virus elementary bodies.

Study of the sections revealed many cells with either intranuclear (Fig. 6 *a*) or cytoplasmic (Fig. 6 *b*) inclusion bodies, but none in which both types of inclusion bodies were present.

From the findings described above, it is evident that the inunction of two viruses among normal corneal cells is followed by both single and multiple virus infections of individual cells. Thus, following infection by a mixture of vaccinia and B viruses, multiple virus infections of single cells were discovered in two of four rabbits, while all four rabbits yielded evidence of single virus infections of cells by one or the other of the two viruses employed.

Simultaneous Infection of the Normal Cornea with Vaccinia and Herpes Viruses

In order to confirm and extend the findings which had been obtained in the first experiment, herpes virus was substituted for the more necrotizing B virus in Experiment 3.

Experiment 3.—Three domestic rabbits were used. The mixture of viruses consisted of equal parts of a 1 per cent testicular suspension containing vaccinia virus, and a 10 per cent brain suspension containing herpes virus.

Rabbit E: This rabbit was killed 48 hours after inoculation. At that time an early but definite keratitis in the right eye was evident, whereas the linear scratches on the left cornea presented but a negligible inflammatory reaction.

Histopathological examination showed increased stratification of the epithelium along each line of scarification and an infiltration of mononuclear and polymorphonuclear cells. No evidence for specific virus infection was discovered.

Rabbit F: This rabbit was killed 48 hours after inoculation. The findings were essentially the same as for rabbit A, except that examination revealed numerous Guarnieri bodies. No evidence for specific infection by herpes virus was found.

Rabbit G: This rabbit, killed 72 hours after inoculation, showed an extensive keratitis in the right eye, but no visible reaction in the left cornea. Serial sections were prepared from the right cornea. Examination of these sections revealed extensive epithelial proliferation at the sites of scarification, cellular infiltration, and, at one site, vesicle formation. Numerous Guarnieri bodies and a few intranuclear inclusions were seen. The two types of inclusion bodies occurred as separate groups at different sites along the lines of scarification except in a single place where both types were present in adjacent cells and in the same cell (Fig. 4).

The results of Experiment 3 showed that vaccinia virus and herpes virus in combination can simultaneously infect a single cell, for in one of the three host animals both an intranuclear inclusion body and a cytoplasmic inclusion body were present within a single cell.

Simultaneous Infection of the Skin and Testicle with Virus III and Vaccinia Virus

Experiment 4 was planned to determine whether cells other than those of the anterior cornea would lend themselves to simultaneous infection by several viruses. Normal rabbit skin and testicle were used to provide host cells for the inoculation of two viruses,—virus III and vaccinia virus. Virus III, the pathogenicity of which had been enhanced by repeated testicular passage immediately before use, was selected as the virus to bring about the formation of intranuclear inclusion bodies, and vaccinia virus, which had been successfully used on previous occasions, was again utilized in an attempt to produce readily recognizable cytoplasmic inclusion bodies.

Experiment 4.—Two male domestic rabbits, rabbits H and I, were used. The virus mixture consisted of a 1 per cent testicular suspension containing vaccinia virus mixed with equal parts of a 10 per cent testicular suspension containing virus III. Each of four successive decimal dilutions (10^1 through 10^4) of this mixture was used in 0.2 ml. amounts for endermic inoculation at four sites and for the injection of the four testicles of the two rabbits, 0.25 ml. being injected into each testicle.

When killed 4 days after inoculation, both rabbits showed definite inflammatory reactions at each site of injection. Of the eight skin sites injected, the histopathological findings of interest were confined to a single lesion which is the only one that will be considered here. This lesion resulted from the injection of the 10^2 dilution. It showed a few cells which contained Guarnieri bodies. These cells were surrounded by areas of necrosis, but there was little evidence of cellular infiltration. The testicles revealed numerous necrotic areas interspersed among apparently normal tissues. These areas were attributed to the effects of infection by vaccinia virus. Sections prepared from the testicles that had received the 10^2, 10^3, and 10^4 dilutions of the virus mixture revealed intranuclear inclusion bodies of type A in the interstitial cells, a reaction characteristic of virus III infection.

No evidence of simultaneous infection of a single cell by both viruses was found.

Our failure to demonstrate multiple virus infection of single cells in this experiment might conceivably be attributed to the use of cells less suitable to the development of inclusion bodies than those of the cornea, or, and this seems more probable, the necrotizing activity of the vaccinia virus may have prevented, or at least masked, the intracellular reaction characteristic of virus III infection.

Simultaneous Infection of Tissues Immune to Myxoma Virus by Three Viruses: Myxoma Virus, B Virus, and Vaccinia Virus

Experiment 5 was planned in a further attempt to demonstrate multiple virus infection of cells other than those of the cornea, the rabbit papilloma, and the developing chick embryo (6). Cottontail rabbits were utilized which had been rendered hyperimmune to myxoma virus by repeated reinoculation with large amounts of it. Three viruses were introduced simultaneously: myxoma virus, to bring about an immediate and heightened local response, and vac-

cinia and B viruses, capable of producing, when together, both cytoplasmic and intranuclear inclusions within single cells. The following experiment was carried out.

Experiment 5.—Ten per cent suspensions of myxoma, B, and vaccinia viruses were prepared and mixed in equal parts. Portions of the resulting mixture, undiluted and diluted 1 to 10, were used in 0.5 ml. amounts for the infiltration of a skin site and of one testicle on each of two cottontails known to be hyperimmune to myxoma virus.

Rabbit J: This rabbit was killed 4 days after inoculation. The cutaneous sites of inoculation showed a marked inflammatory reaction with central necrosis and surrounding edema. The testicles, which were twice the normal size, were also markedly inflamed and necrotic. Examination of sections revealed that all evidence of specific virus activity was limited to the cutaneous area that had received the 10 per cent dilution. In this area, intranuclear inclusion bodies of type A and Guarnieri bodies were seen, thus establishing the presence of both B and vaccinia viruses. In no single cell, however, were both cytoplasmic and intranuclear inclusion bodies noted. All the sections of testicular tissue revealed an extensive necrotizing inflammatory reaction, but no evidence for specific virus activity. Moreover, in no instance were inclusions of the type which often accompany infection by myxoma virus found.

Rabbit K: This rabbit, which was killed 5 days after inoculation, showed a much more extensive inflammatory reaction than rabbit J. This was evidenced by generalized polymorphonuclear invasion and abscess formation. There was a complete absence of inclusion bodies suggestive of virus activity.

It is not surprising that detectable inclusion bodies were not present following the simultaneous introduction of the three viruses, *viz.* myxoma, B, and vaccinia viruses, when the extensive necrosis which occurred at the sites of injection is taken into consideration. The usual necrobiotic effects of B and vaccinia viruses undoubtedly were so exaggerated by the accelerated and heightened response of the hyperimmune tissues to reinjection with myxoma virus that any inclusion bodies were obscured or destroyed. Moreover, the tissues infected in this experiment were the same as in the preceding experiment in which we also failed to demonstrate multiple virus infection of individual cells.

DISCUSSION

The present observations establish the fact that a single epithelial cell can be simultaneously infected by two viruses. The evidence is found in the repeated demonstration that the epithelial cells of the rabbit's cornea can respond to two viruses (of which one has the capacity of forming inclusion bodies within the nucleus, the other of producing them within the cytoplasm) by the formation of both intranuclear and cytoplasmic inclusion bodies within individual cells.

Anderson (6), moreover, by utilizing different combinations of viruses corroborated our earlier findings (1, 5) and procured results similar to those reported in the present paper. She established cytological evidence for the

dual parasitization of single chick embryo cells by two viruses that yield distinctively different inclusion bodies. The combinations of viruses used were fowl pox and laryngotracheitis, fowl pox and herpes simplex, herpes simplex and vaccinia, and herpes simplex and rabies. It is of interest that experiments involving the combination of fowl pox and vaccinia, two viruses that result in cytoplasmic inclusion bodies, were unsuccessful.

From the findings set forth, it is plain that the results of the present investigations substantiate our previous studies (1). The observations made in the earlier work led us to conclude that coexistent virus infections had been produced in the cells of a virus-induced tumor, Shope's rabbit papilloma. This conclusion was based on experimental evidence that the inoculation of a mixture of two viruses into a papilloma had resulted in the presence within single cells of two sorts of inclusion bodies, cytoplasmic and intranuclear, characteristic of the viruses respectively. Since all of the cells of an actively growing papilloma presumably contain papilloma virus, the presence of these inclusions would seem to indicate the coexistence of three viruses within an individual cell.

As previously stated it has been often assumed that a single cell can be infected by but a single virus. This assumption has been based on the intimate type of parasitism that characterizes viruses—on the belief that the presence of one virus within a cell would so alter the characteristics of that cell that another virus could not be active there at the same time. This belief is no longer tenable, at least for some of the viruses with which Anderson (6) and ourselves have worked. The question remains valid, nevertheless, as concerns the viruses that have been implicated in the phenomenon known variously as "sparing effect," "interference," or "cell blockade," a situation in which one strain of a virus modifies or prevents infection by a second, usually more pathogenic, strain. Some workers have assumed that the "interfering" virus exerts an "antagonistic" effect on the other virus, thereby protecting the host cell against the more serious infection. This hypothesis first found support from experimental studies with plant viruses. These investigations (7–9) suggested that closely related strains of a single virus could not simultaneously occupy the same plant tissue. Since this antagonism did not concern immunologically distinct viruses, the phenomenon might represent a rapidly acquired specific resistance induced by related strains of virus, resulting in an infection with modified or slight manifestations. Moreover, the demonstration of one virus in the presence of resistance to a second, more pathogenic but related, virus does not necessarily mean that all of the cells are occupied by the first virus to the exclusion of the second. It is equally possible, perhaps probable, that a carrier state may coexist with a state of acquired immunity. This explanation might apply where the "interference" phenomenon has been observed in infections with animal viruses (10–19). In four instances involving different viruses (12,

13, 15, 19), however, the manifestations of an otherwise serious virus disease were obscured after two apparently unrelated viruses had been inoculated within a few hours.

Many hypotheses have been advanced to explain the intimate type of parasitism characteristic of so many virus infections, to account for the sharply defined host specificity and tissue affinity so frequently observed, and to make understandable the mechanisms whereby a virus can multiply within its host cell. These hypotheses have been commonly predicated on the "lock and key" concept that the virus "fits" into the enzyme systems of the host cell, perverting the cell's metabolism, such perversion resulting in the various manifestations of deranged cellular activity and cellular destruction characteristic of most virus infections. It is reasonable to suppose that when two or more viruses simultaneously exhibit their characteristic activities within a single cell, they "fit" into the intracellular mechanisms at different "points;" whereas when they compete for the same "point," the "blockade" or "interference" phenomenon is observed. Many analogies from the fields of enzymology, pharmacology, and immunology come immediately to mind. One may perhaps hopefully expect that further study of multiple virus infections of single cells will help in elucidating not only the phenomenon of "interference," but also the nature of intracellular virus activity.

SUMMARY

Evidence is presented to show that two or more viruses can simultaneously manifest their characteristic activities within individual epithelial cells of the normal rabbit's cornea. This evidence, together with that previously presented (1, 5, 6), makes plain that multiple virus infection of a single host cell can take place in corneal cells, in the cells of chick embryos, and in those of rabbit tumors, both benign (Shope's papilloma) and malignant.

Certain implications of the findings are discussed.

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EXPLANATION OF PLATES

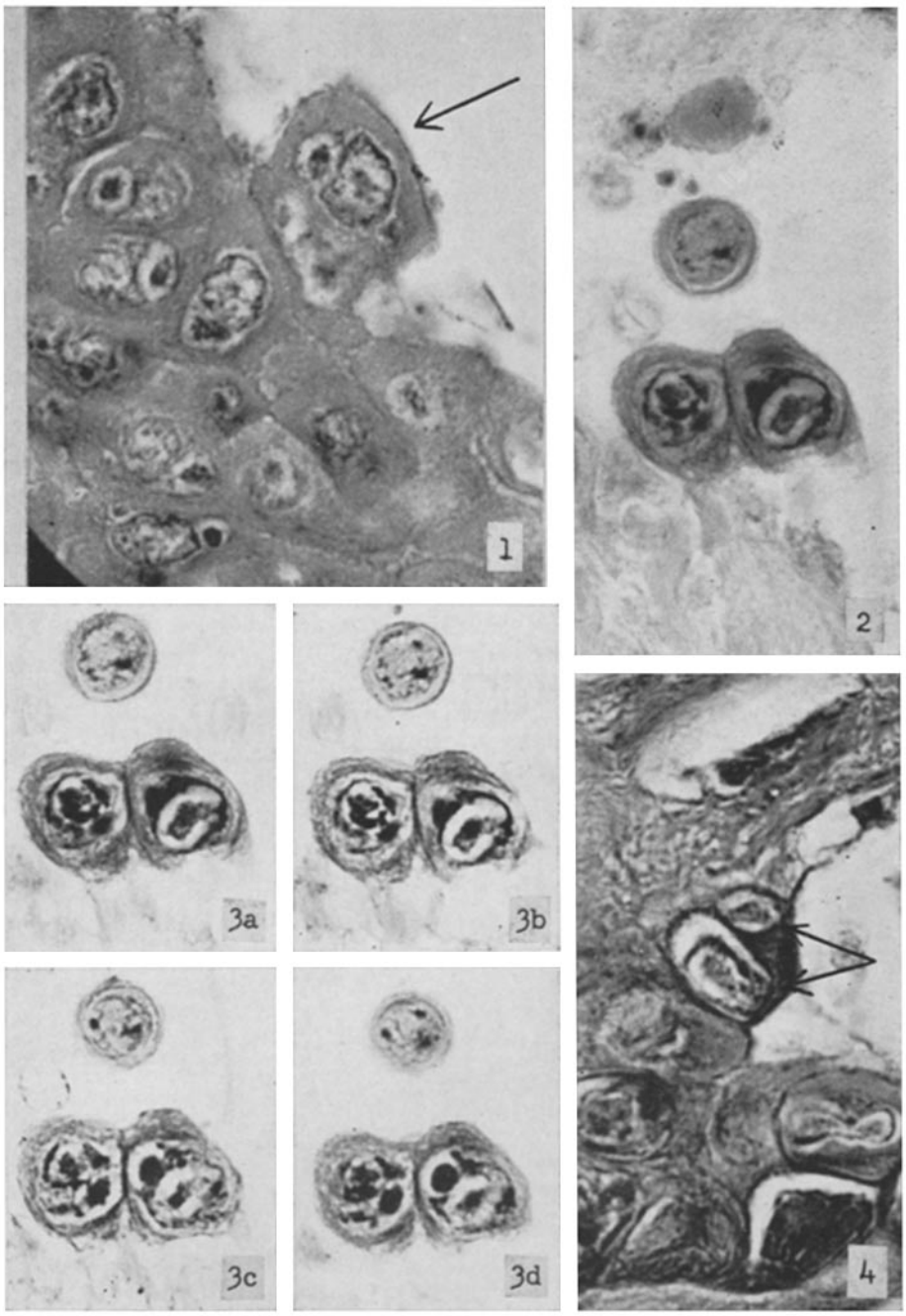
PLATE 15

The photographs were made by Mr. Merwyn C. Orser from sections stained by Giemsa's method.

FIG. 1. Portion of a section of cornea from rabbit A originally inoculated with a mixture of vaccinia virus and B virus in suspension. The cell designated by the arrow shows clearly the presence of a type A intranuclear inclusion body of B virus infection and a cytoplasmic inclusion body of vaccinia virus infection. × 1500.

FIGS. 2 and 3. Section of cornea from rabbit C which was killed 72 hours after a mixture of vaccinia virus and B virus was inoculated into the scarified cornea. Two cells well separated from all adjacent cells are shown. Within each of these cells will be seen a Guarnieri body and a type A intranuclear inclusion body. By using an oil immersion lens and focusing at four successive levels, four photographs were taken (*a*, *b*, *c*, and *d* of Fig. 3), which show unequivocally the singleness of each cell and the presence within each cell of both a cytoplasmic inclusion body and an intranuclear inclusion body. Fig. 2, × 1500; Fig. 3, × 1250.

FIG. 4. Section of cornea from rabbit G which was killed 72 hours after inoculation with a mixture of vaccinia virus and herpes virus. A Guarnieri body and a type A intranuclear inclusion body within a single cell are indicated by arrows. × 1500.



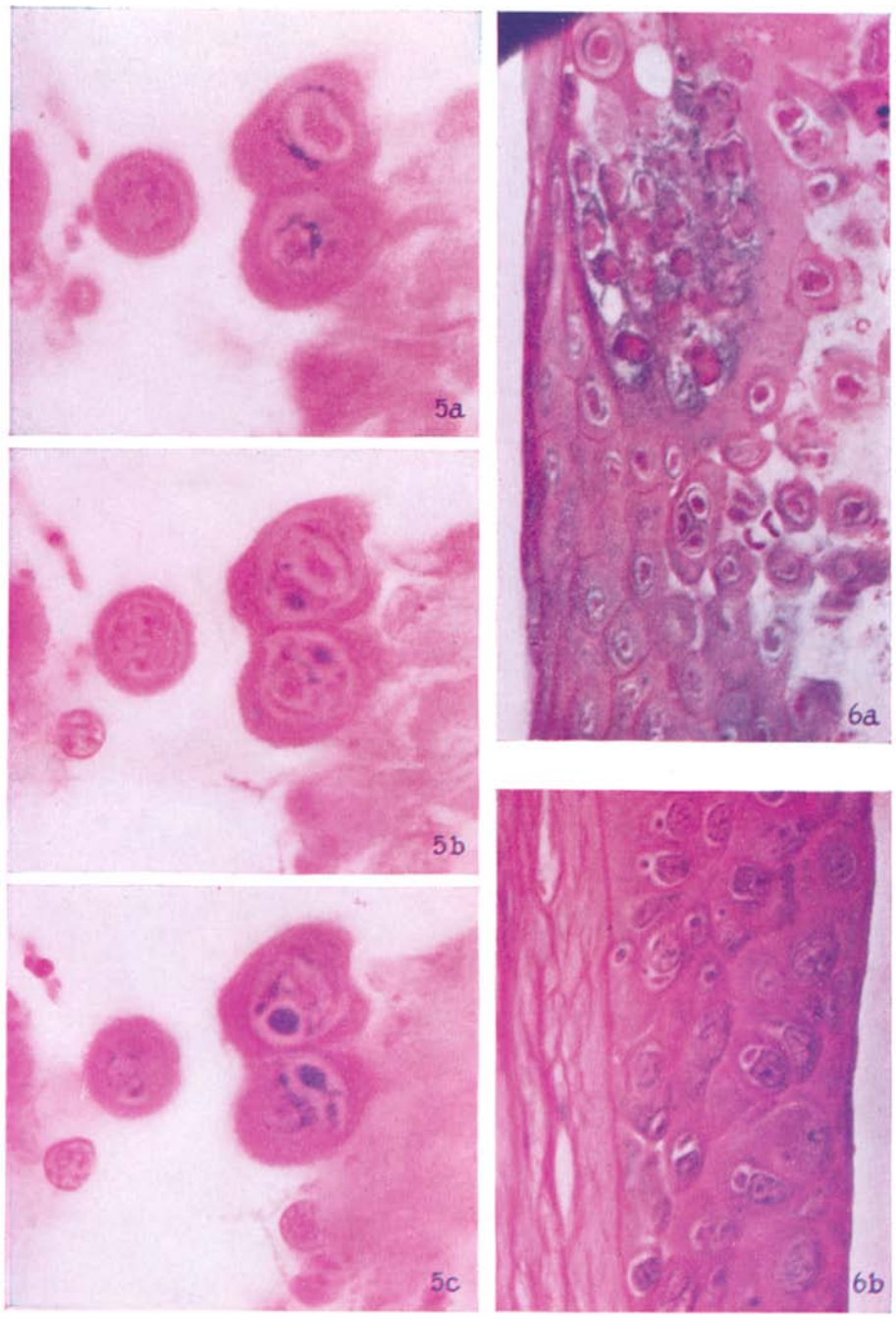
(Syverton and Berry: Multiple virus infection of single cells)

PLATE 16

These prints were made by Mr. Adrian Ter Louw and Mr. Charles Brownell who employed a modification of the Kodak wash-off relief process for separation negatives that were exposed directly, from sections stained by Giemsa's method.

FIG. 5. The two cells depicted in Fig. 3 are shown in color. The three photographs were made by using an oil immersion lens and focusing at three successive levels. × 1500. Fig. 5 *a* shows the type A intranuclear inclusion bodies in sharp focus; Fig. 5 *b*, an intranuclear inclusion and a cytoplasmic inclusion body in each cell but none of the inclusion bodies in sharp focus; Fig. 5 *c*, the granular cytoplasmic inclusion bodies are in sharp focus.

FIG. 6. Sections of cornea from rabbit D which was killed 72 hours after inoculation to show in color type A intranuclear inclusion bodies (Fig. 6 *a*) and cytoplasmic inclusion bodies (Fig. 6 *b*). × 600.



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