

FATE OF MUMPS VIRUS IN THE EMBRYONATED EGG AS
DETERMINED BY SPECIFIC STAINING WITH
FLUORESCHEIN-LABELLED IMMUNE SERUM*

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PLATES 32 AND 33

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The adaptation of mumps virus to the developing chick embryo (1-3) provided a readily accessible host for the study of this agent. Despite the considerable amount of work which has been done, however, very little is known regarding the sites of virus multiplication within the infected egg. This is attributable to the fact that although mumps virus is on occasion capable of killing the embryo, it does not produce any gross or microscopic lesions which can be specifically associated with the infection (1, 3-5). To demonstrate its presence, therefore, indirect methods have necessarily been employed.

Using complement fixation to test the tissues and fluids of embryos inoculated into the amniotic, chorioallantoic, and yolk sacs, Habel (1) found that the virus was widely distributed throughout the embryo and its membranes although the concentration was greatest in the cavity inoculated. Similar results have been reported by Enders (6) who stated that after a number of passages the virus becomes adapted to the embryo and can be recovered from most of its tissues and fluids. The demonstration of mumps hemagglutinins (2) provided an additional method, which was simpler than complement fixation, for detecting infection. Hemagglutinins have been found in high titer in the extraembryonic fluids but they have not been consistently demonstrable in the tissues (7).

A new approach to the problem was made possible recently by the successful use of fluorescein-labelled immune serum as a specific stain to visualize mumps virus antigen directly within the infected cell (8-10). The present report deals with the use of this technic in a study of the progress of mumps virus infection following its intraamniotic inoculation into the developing chick embryo. The immune serum employed was obtained from monkeys convalescent from experimental mumps. This serum was conjugated with fluorescein isocyanate and used as a histochemical reagent on tissue sections of a series of embryos in various stages of infection. The specific conjugate formed a precipitate over

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those areas where the virus antigens were located. The unreacted portions of the serum were then washed away and the sections examined under the fluorescence microscope. The results based on the specific staining of the tissues were compared with the hemagglutinin and infectivity titers of the corresponding extraembryonic fluids.

Materials and Methods

Stock Virus and Control Material.—The stock virus and control material used for injection consisted of pooled allantoic fluids. The stock virus was derived from the sixty-second egg passage of the Enders strain of mumps. The control material was obtained from normal eggs which had been incubated under similar conditions. The fluids were stored in small amounts in glass sealed test tubes in a dry-ice cabinet. On thawing, the stock virus titered 1:640 for hemagglutinins and had an ID_{50} of 7.0 when tested according to the methods to be described below for the amniotic fluids harvested.

Inoculation of Eggs.—Groups of 3 to 5 dozen 8 day old white Leghorn chick embryos were inoculated by the amniotic route with 0.1 ml. amounts of 10^{-2} dilutions of the infected and uninfected allantoic fluids. One experiment was carried out using 16 day old embryos. The eggs were incubated at 35°C. and candled daily for records of mortality.

Harvesting of Tissue and Fluids.—Immediately following inoculation and at daily intervals thereafter duplicate eggs were removed from each series and samples of allantoic and amniotic fluids were withdrawn by means of separate Pasteur pipettes. The embryos were emptied into Petri dishes, dissected away from the extraembryonic membranes and placed on narrow strips of cardboard along with representative portions of each of the membranes. The cardboard strips were transferred to rubber stoppered test tubes of appropriate size, quick frozen at -70°C . and stored at -20°C . The amniotic and allantoic fluids were then tested for the presence of hemagglutinins and the remaining material was frozen and stored according to the method used in the preservation of the stock virus. The tissues and fluids obtained in this way represented a daily sampling of the two series of embryos from the time immediately after inoculation through hatching.

Sectioning and Staining of the Tissues.—Frozen sections of the embryos and membranes were prepared according to the method of Linderstrøm-Lang and Mogensen (11) as modified by Coons and his associates (13). In the initial experiments the embryos were cut at various levels to determine the localization of the virus within the tissues. On the basis of the results obtained two transverse planes of sectioning were selected for routine use: one through the head in the region of the lower quarter of the eyes; the other through the body approximately half-way down the trunk. The frozen blocks of the extraembryonic membranes, which consisted of numerous folds of the thin collapsed tissues, were cut at random. The sections were mounted and dried, fixed in acetone for 15 minutes, then placed in a 37°C. incubator to evaporate the acetone, following which they were ready for staining.

The serum used in the preparation of the specific conjugate was obtained from two monkeys which had at one time survived experimentally induced mumps and which received periodic booster doses of infected monkey parotid gland antigen. The sera of several bleedings were tested for hemagglutinin inhibition and complement fixation, then pooled. The titers obtained ranged from 1:2048 to 1:32,768 for hemagglutinin inhibition and 1:64 to 1:1024 for complement fixation. The pooled serum was then concentrated (13), conjugated with fluorescein isocyanate, and purified as previously described (12).¹ Before being used as a stain the con-

¹ We are greatly indebted to Dr. Albert H. Coons for the preparation of the conjugate and for his many helpful suggestions throughout the course of this work.

jugate was absorbed twice with chicken liver powder. This, as well as the staining of the sections and the tests for the specificity of the staining, was carried out as described in reference 12.

After the sections had been studied under the fluorescence microscope the coverslips were floated off and the tissues were fixed in formalin and stained with hematoxylin and eosin. They were then examined for evidence of pathology.

Hemagglutinin and Infectivity Titrations.—As time permitted, quantitative hemagglutinin titrations were carried out with all the infected amniotic fluids harvested, using serial twofold dilutions. On the days the material was thawed these fluids were also inoculated in 0.1 ml. amounts of serial tenfold dilutions into groups of six 8 day old embryonated eggs. The route of inoculation was the amniotic sac and the period of incubation was 6 days at 35°C. The amniotic control fluids and the infected and uninfected allantoic fluids were tested for hemagglutinins and infectivity in 1:10 and 1:100 dilutions respectively. The procedures used in carrying out the tests were those described by Enders and Levens (5). The ID_{50} calculations were based on the formula of Reed and Muench (14).

EXPERIMENTAL

Localization of Mumps Virus Following Intraamniotic Inoculation into 8 Day Old Chick Embryos

As determined by specific staining, the localization of mumps virus following intraamniotic inoculation into 8 day old chick embryos was restricted to those cells which came into direct contact with the inoculum. During the early stages of the infection these included the cells lining the amniotic sac, and the epidermal and pharyngeal epithelium. Since it is virtually impossible to enter the amniotic cavity without contaminating some of the structures which surround it, a small amount of virus was probably introduced into the allantoic cavity and occasionally into the extraembryonic celom. With time this virus became detectable in the cells lining the chorioallantoic membrane and less frequently in the peritoneum. During the later stages of the infection the yellow green fluorescence was also seen in the lumina of the trachea, bronchi, and parabronchi of the lungs, and in the gastrointestinal tract. This specific material was principally if not entirely extracellular. That the reaction observed with the specific conjugate in the regions mentioned was directly attributable to the presence of the virus or its antigenic components was indicated by the facts: (a) that it could not be elicited in the corresponding control sections of embryos inoculated with normal allantoic fluid; and (b) that it was readily inhibited by preliminary treatment of the sections with unconjugated homologous immune serum but not with normal serum.

Staining which could *not* be attributed to the presence of the virus was seen in embryonic cartilage and in the yolk sacs of the younger chicks. Although it varied in intensity from embryo to embryo, this staining was detectable in both the infected and uninfected tissues and was not specifically inhibited by unconjugated homologous immune serum.

The progress of the infection will be considered by describing the staining

seen in the sections of successive embryos in the order of its appearance. The results are summarized in Table I in which the relative staining of the tissues of a representative series of embryos is graded on the basis of 1 to 4.

Amnion.—In the amniotic membrane the presence of the virus was first detectable 1 to 2 days after inoculation. The earliest sign of infection was the appearance of brightly fluorescent granules of varying size located in occasional cells lining the membrane (Fig. 1). These granules gradually increased in number and at the same time tended to become arranged in circles and semi-circles in the cytoplasm. As they grew in size, they coalesced, filling the cytoplasm of the cells. With the exception of faint stippling and an occasional

TABLE I
Relative Staining of the Tissues of a Representative Series of Embryos throughout the Course of the Infection

Day after inoculation	Amnion	Skin and oropharynx	Nasopharynx	Chorioallantois	Peritoneum
0	—	—	—	—	—
1	1+	±	—	—	—
2	2+	1+	—	—	—
3	3+	2+	±	1+	1+
4	4+	3+	1+	2+	—
5	4+	4+	2+	2+	—
6	3+	3+	3+	—	2+
7	2+	3+	3+	3+	—
8	2+	2+	3+	—	—
9	2+	2+	3+	3+	2+
10	1+	1+	2+	3+	—
11	1+	±	2+	3+	—
13	—	—	±	—	—

± faint stippling. 4+ maximum staining.

granule, no staining was seen in the nuclei. As the infection progressed, more and more cells became involved until the entire surface of the amnion formed an uninterrupted line of specific fluorescence one or two cell layers deep (Fig. 2). In most of the fields examined, only one surface of the membrane showed the presence of the virus (Fig. 2). However, a few stretches of membrane were seen in which the cells on both sides were stained (Fig. 3). Whether these were folds in the membrane or whether they were areas in which the amnion and chorioallantois were fused was not determined.

The extent and brightness of the staining reached a peak 3 to 5 days after inoculation. At this time areas were visible in which some of the presumably infected cells seemed to be disintegrating while others appeared as pale green ghost-like structures containing a few brightly fluorescent granules. As the infection ran its course this type of staining became increasingly more evi-

dent with fewer and fewer intact fluorescent cells clearly discernible. By the 7th to the 9th day of the infection most of the staining appeared either as brightly fluorescent extracellular granules and amorphous material or as pale green cellular debris. Some of this adhered to the surface of the membrane whereas some was trapped in the deeper layers of tissue. Thus, as the infection waned the zone of staining became broader, fainter, and less well defined cytologically (Fig. 4).

Skin and Oropharynx.—With minor differences the staining of the epidermal and buccal epithelium followed the same pattern as in the amniotic membrane. The virus was first detectable 2 to 3 days after inoculation as brightly fluorescent intracytoplasmic particles of varying size. As these particles grew in size and number they gradually filled the cytoplasm of the cells (Fig. 5). Since the lumen of the pharynx in the younger embryos was barely discernible at the level sectioned, the contiguous layers of epithelium appeared first as an interrupted, later as a solid line of staining approximately 1 to 2 cell layers deep (Figs. 6 and 7). The staining reached a peak 4 to 5 days after inoculation and thereafter declined progressively. This coincided with a gradual increase in the thickness of the buccal epithelium and with the formation of feathers in the skin. The deposition of the fluorescent precipitate became discontinuous, fainter, and ill defined as some of the cells disintegrated or were sloughed off into the adjoining cavities. Those which remained intact flattened out and became incorporated, individually or in groups, into an acellular horny layer which for a time retained its yellow green fluorescence, then faded gradually (Fig. 8). By the 10th day of the infection very little virus could be detected in either the skin or oral pharynx (Fig. 9).

Nasopharynx.—From its initial presence in the buccal epithelium the detectable antigen gradually extended to the cells lining the upper and more remote portions of the pharynx and by about the 8th day of the infection outlined the entire structure. In the nasal and supraorbital sinuses the staining seen at this time appeared thicker and less discrete. This was due to the presence of numerous folds in the epithelium in which the cellular localization of the virus was poorly defined (Fig. 10). Lower down, the deposition of the specific conjugate was more like that in the epidermal and buccal epithelium (Fig. 11) although progressively fewer intact infected cells were clearly visible (Fig. 12). Some fluorescent material was also detectable in the subcutaneous tissue immediately below the epithelium (Figs. 11 and 12). As the infection decreased, more and more of the virus appeared extracellularly. The lumen of the pharynx became filled with brightly fluorescent granules and cellular debris (Fig. 9) which were apparently swallowed periodically. At the time of hatching only an occasional granule was seen here and there over the surfaces which had been previously affected. In two of the chicks, faint granular staining was detectable in a few restricted foci in the subcutaneous tissue.

As the embryos matured, brilliant fluorescence appeared in the mucus secretion, and in the oral glands in sections of the infected tissues and to a lesser extent in those of the uninfected tissues. Since this staining in infected embryos was partially inhibited by preliminary treatment of the sections with unconjugated homologous immune serum, some of it may have been specific.

Chorioallantois and Peritoneum.—After about the 3rd day of the infection varying amounts of virus were detectable in the cells lining the chorioallantoic membrane. No virus was present, however, in two of the membranes harvested 6 and 8 days after inoculation. The deposition of the fluorescent precipitate was clearly intracytoplasmic although the staining was generally not as advanced as in the corresponding sections of the other tissues. In the chorioallantois, the localization of the virus was restricted to a discrete line of cells on one surface of the membrane only. Similar staining of the cells of the peritoneum was seen in three of the embryos harvested 3, 6, and 9 days after inoculation. The presence of the virus in the allantois and peritoneum appeared to be determined by the extent of the contamination of the allantoic cavity and extraembryonic celom in the course of inoculation.

Respiratory and Gastrointestinal Tract.—In the older embryos large masses of fluorescent material were periodically observed in the esophagus and gizzard. The greater proportion of this staining was clearly extracellular although a few brightly fluorescent dots were occasionally present in the cells lining the inner walls of these organs. After the 7th day of the infection extensive staining was regularly seen in the lumina of the trachea, bronchi, and parabronchi of the lungs. The sudden appearance, random distribution, and ill defined cellular localization of the specifically reacting material suggested that it consisted of virus-containing cell debris which had been inhaled from the pharynx following the dissolution of the tracheal plug.

Hemagglutination and Infectivity of the Extraembryonic Fluids

The hemagglutination and infectivity of the amniotic and allantoic fluids of duplicate embryos harvested at daily intervals are compared with the relative staining of the corresponding membranes in Table II. As indicated, the amount of virus present in the fluids clearly paralleled the staining of the tissues. Thus, coincident with the appearance of the early type of staining in the amniotic membrane, a slight increase in infectivity was detectable in the amniotic fluid of one of the embryos obtained 1 day after inoculation. As more and more of the cells became infected, the amount of virus present in the fluids rose sharply and by the 3rd day of incubation reached a level at which hemagglutinins were detectable. During the next 2 days of the infection both the staining of the tissues and the infectivity of the fluids reached a maximum and thereafter declined gradually. Since the titer of hemagglutinin did not vary significantly throughout the latter period the results suggested that although the propor-

tion of infectious to non-infectious virus decreased progressively, the total amount of virus present in the fluid remained more or less constant. This was

TABLE II
Hemagglutination and Infectivity of Amniotic and Allantoic Fluids as Compared with the Staining of the Corresponding Membranes

Day after inoculation	Egg No.	Amniotic fluids		Staining of amniotic membrane	Allantoic fluids			Staining of chorioallantoic membrane
		HA	ID ₅₀		HA	Infectivity		
						1:10	1:10	
Start	1	—	1.5	—	—	2/4*	—	—
	2	—	1.6	—	—	—	—	—
1	1	—	2.4	1+	—	1/6	—	—
	2	—	2.0	—	—	0/6	—	—
2	1	—	4.7	2+	—	—	—	—
	2	—	4.5	—	—	—	—	—
3	1	320	>7.0	3+	—	6/6	—	1+
	2	160	6.7	3+	—	—	6/6	2+
4	1	40	7.7	4+	—	—	4/4	2+
	2	640	7.3	—	—	—	4/4	—
5	1	640	7.5	4+	—	—	—	2+
	2	80	7.6	4+	+	—	—	3+
6	1	—	—	—	—	—	—	—
	2	160	6.7	3+	—	—	—	—
7	1	320	<7.0	2+	+	—	—	3+
	2	160	6.8	2+	+	—	—	3+
8	1	40	6.3	2+	—	—	—	—
	2	160	7.6	3+	+	—	—	3+
9	1	320	5.5	2+	+	—	—	3+
	2	160	5.2	2+	+	—	—	3+
10	1	—	<4.0	1+	—	—	—	3+
	2	±	<3.0	1+	±	—	—	3+

* Ratio of eggs infected to eggs tested following inoculation with the dilutions indicated.

borne out by the type of staining seen during the later stages of the infection which consisted more and more of extracellular virus and cell debris. As in the amniotic membrane, the appearance of the staining in the chorioallantoic membrane was reflected by an increase in the infectivity and, during the later

stages of the infection, by the appearance of hemagglutinins in the allantoic fluids. The results based on the hemagglutination and infectivity of the fluids are in agreement with those of Ginsberg, Goebel, and Horsfall (15) and others (16, 17). As in the present experiments, the hemagglutinin titers of individual eggs were found to vary widely.

Mortality and Pathology

No differences were observed in the mortality of the infected and control series of embryos throughout the period of incubation. Nor were there any gross or microscopic lesions present which could be specifically attributed to the infection. The sloughing off of the surface epithelium and the presence of cell debris in the adjoining cavities seen in the sections of the infected series of embryos was also detectable in many of the sections of the uninfected embryos. The temperature of incubation was so unfavorable, however, that at the time of hatching only a few of the chicks succeeded in extricating themselves from their shells and none survived for more than 3 days thereafter.

Infection of 16 Day Old Embryos

In the experiment in which 16 instead of 8 day old embryos were used the ID_{50} of the amniotic fluids immediately after inoculation was 3.7. After 4 days of incubation, it was 3.3. The tests for hemagglutinins were negative on both occasions and no staining was seen in any of the tissues. This evidence indicated that the older embryos were incapable of supporting the growth of the virus.

DISCUSSION

In the embryonated egg the virus of mumps grows for a relatively long period of time without producing any histologically apparent tissue damage (1, 3, 5). The evidence presented indicates that the multiplication is restricted to the cells of those surfaces which come into direct contact with the infected fluid. Following intraamniotic inoculation these include the cells lining the amniotic sac, the epidermis, and the pharynx with its various ramifications. When, in the course of intraamniotic inoculation, the contamination of the allantoic cavity or of the extraembryonic celom was apparently sufficient, the virus was also present in the lining of the chorioallantoic membrane and in the peritoneum.

Specific staining of the tissues bordering the amniotic cavity was first detectable 1 to 2 days after inoculation. It reached a maximum 4 to 6 days after inoculation and thereafter decreased progressively. This sequence of events paralleled rather closely the rise and fall in the infectivity of the amniotic fluids. The presence of hemagglutinins, on the other hand, provided a less sensitive measure of virus multiplication than did the staining.

Contrary to previous reports of a widespread distribution of the virus (1, 6), no specifically stained material was found in any of the other tissues examined. Thus, *if* the virus is picked up by the blood stream, it is apparently incapable of invading the embryo by this route. In this connection, Beveridge, Lind, and Anderson (3) were unable to establish the infection following intravenous inoculation of the virus. In tissue cultures, on the other hand, the virus has been propagated in a variety of chick tissues (10, 18). Here, as in the embryo, the multiplication of the virus was restricted to the cells on the surface of the individual tissue fragments (10) which were bathed by the infected fluid.

From the earliest appearance of the brightly fluorescent granules, through the stage in which these granules gradually filled the cytoplasm of the cells, the staining seen was such as to suggest multiple foci of infection within each cell. This could be interpreted to mean either that the cells were infected with more than one virus particle or that one virus particle was capable of initiating the growth of several "virus colonies." Since a rise in the infectivity of the amniotic fluid was detectable at about the same time that the early type of staining appeared in the tissues, and since this took place long before any of the infected cells were seen to be disintegrating, the results suggest that rupture of the cell may not be essential to the liberation of the virus. The protracted period of virus multiplication and the large amounts of virus produced, as compared to its restricted localization, provide further evidence that the same cells may be capable of secreting virus over long periods of time.

To account for the self-limiting nature of the infection and the ultimate disappearance of the virus, two explanations are possible. One is that the supply of susceptible cells becomes exhausted. This is unlikely since in tissue cultures the virus can be propagated for as long as the tissues can be kept alive (19). The other is that the cells are either altered or replaced by less susceptible ones. This is supported by the finding of Beveridge and Lind (20), and others (4, 17), that the older the embryo at the time of inoculation the less sensitive it is to infection. In the present experiments, no virus multiplication could be detected following intraamniotic inoculation of 16 day old embryos.

Preliminary experiments with the PR8 strain of influenza A virus have shown that the distribution of this agent in the embryonated egg is similar to that of mumps with the exception that the epithelium of the respiratory tract is a site of virus multiplication. These studies are being pursued and a detailed account of the findings will be published later.

The author wishes to acknowledge the valuable technical assistance of Jeanne M. Connolly and Sheila Richardson.

SUMMARY

Specific staining with fluorescein-labelled immune serum was used to study the progress of mumps virus infection in a series of embryos harvested at daily

intervals. The results were compared with the hemagglutinin and infectivity titers of the corresponding extraembryonic fluids. The evidence obtained indicated that the multiplication of the virus was restricted to those cells which came into surface contact with infected fluid. Following intraamniotic inoculation into 8 day old embryos, these included the cells lining the amniotic membrane and the epidermal and pharyngeal epithelium. Depending apparently on the extent of the contamination of the allantoic cavity and of the extraembryonic celom in the course of inoculation, varying amounts of virus were also present in the cells lining the chorioallantoic membrane and occasionally in the peritoneum. During the later stages of the infection, staining which was principally if not entirely extracellular was seen in the gastrointestinal tract, and, after the dissolution of the tracheal plug, in the respiratory tract.

The virus was first detected 1 to 2 days after inoculation as brightly fluorescent intracytoplasmic granules of varying size. As these granules increased in size and number they gradually filled the cytoplasm of the outer one or two layers of cells lining the adjoining cavity. The extent and brightness of the staining reached a peak 4 to 6 days after inoculation and thereafter decreased progressively.

The staining of the tissues closely paralleled the rise and fall in the infectivity of the extraembryonic fluids. The development of hemagglutinins, on the other hand, provided a less sensitive measure of virus multiplication than did the staining.

No differences were detected in the mortality of the infected and control series of embryos nor was there evidence of any pathological changes.

In contrast to the 8 day old embryos, no virus multiplication was detected following the inoculation of 16 day old embryos.

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EXPLANATION OF PLATES

The photomicrographs are of tissue sections from chick embryos in various stages of infection. They were taken through the fluorescence microscope after the tissues were treated with homologous immune serum conjugated with fluorescein isocyanate. The lightest areas represent the yellow green fluorescence of the deposited conjugate. The topography of the sections is faintly outlined by the blue-gray autofluorescence of the unreactive portions of the tissues.

PLATE 32

FIG. 1. Folds of amniotic membrane 3 days after inoculation. Early infection. Brightly fluorescent granules of varying size in occasional cells lining the membrane. $\times 140$.

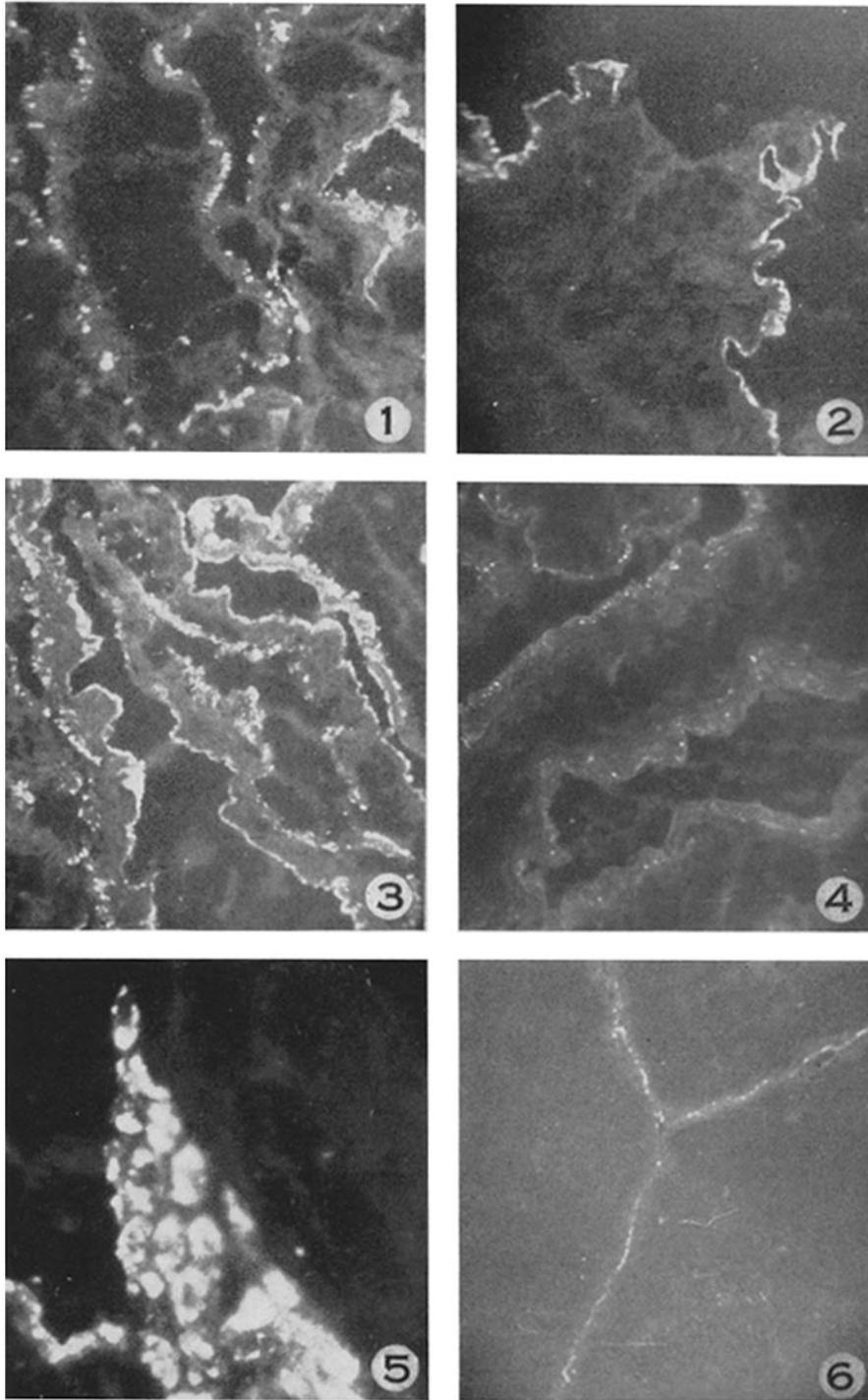
FIG. 2. Amniotic membrane 3 days after inoculation (another section). More advanced infection. Continuous line of staining of epithelial surface of membrane. $\times 140$.

FIG. 3. Amniotic membrane 4 days after inoculation. Staining of both surfaces of membrane. $\times 140$.

FIG. 4. Amniotic membrane 9 days after inoculation. Late infection. Very little virus detectable. $\times 140$.

FIG. 5. Fold of skin at the corner of the mouth (sectioned longitudinally) 4 days after inoculation. Brightly fluorescent granules of varying size in cytoplasm of cells. $\times 560$.

FIG. 6. Oropharynx 3 days after inoculation. Early staining of contiguous layers of epithelium. $\times 140$.



(Watson: Fate of mumps virus in embryonated egg)

PLATE 33

FIG. 7. Oropharynx 4 days after inoculation. Advanced staining of contiguous layers of epithelium. $\times 140$.

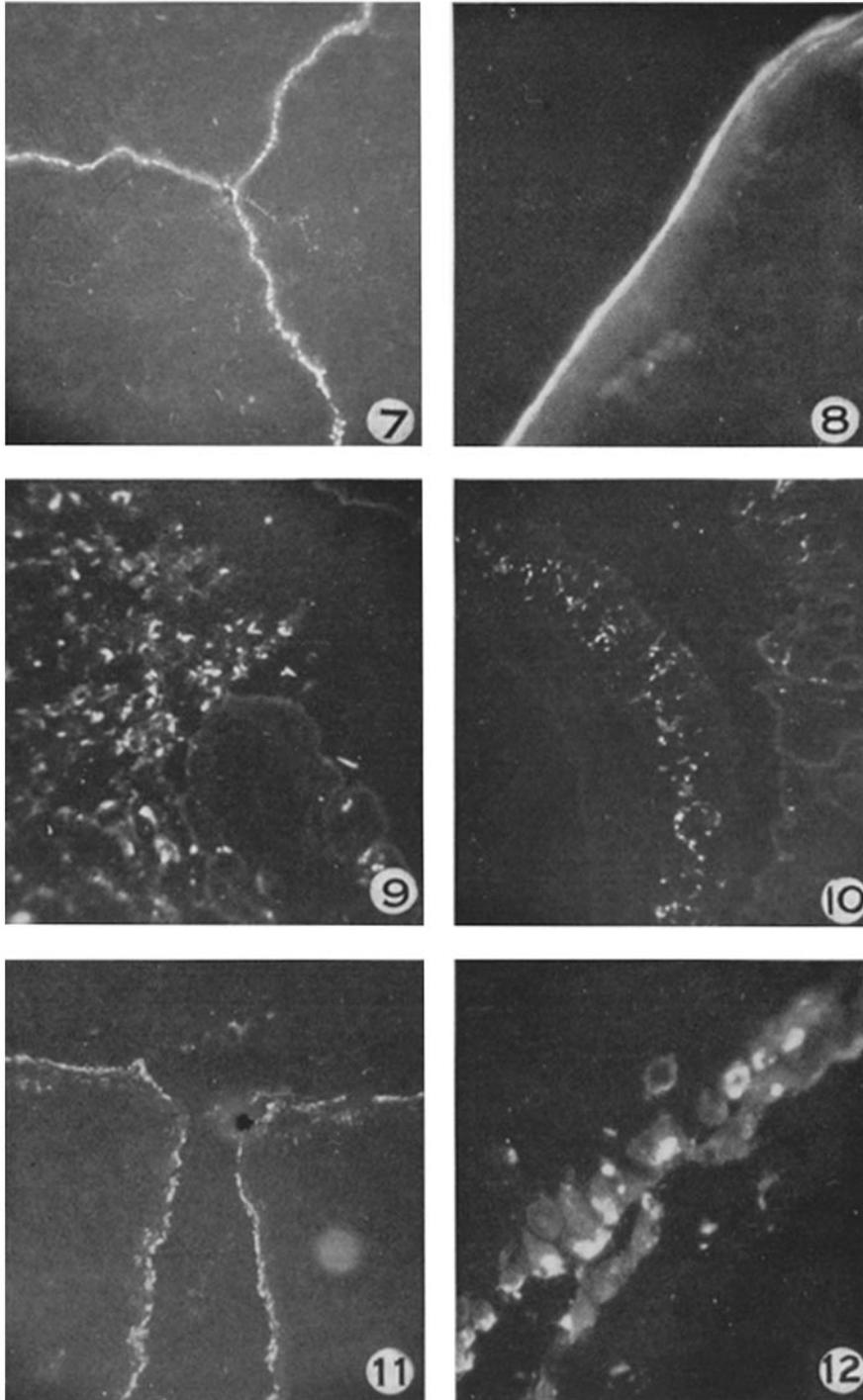
FIG. 8. Corner of the mouth 9 days after inoculation. Staining of acellular horny layer. $\times 140$.

FIG. 9. Oropharynx 11 days after inoculation. Epithelium no longer stained. Fluorescent cells and debris in lumen. $\times 140$.

FIG. 10. Supraorbital sinus 11 days after inoculation. Zone of staining broader due to glandular like invaginations in epithelium in which some of the fluorescent material appears to be trapped. $\times 140$.

FIG. 11. Epithelium in region of cleft palate 9 days after inoculation. Staining like that of oropharynx 4 days after inoculation only fainter and less discrete. $\times 140$.

FIG. 12. Ghost-like cells in region of cleft palate 9 days after inoculation. $\times 560$.



(Watson: Fate of mumps virus in embryonated egg)