his cancer. The methods, previously used successfully to detect a new intranuclear antigen induced by the papovavirus SV40 in transformed human and hamster cells, and in hamster tumors, failed to reveal the presence of immunologically reactive components.

1. Black, P. H., Rowe, W. R., Turner, H. C., Huebner, R. J., Proc. Nat. Acad. Sci., U. S., 1963, v50, 1148.

2. Rapp, F., Butel, J. S., Melnick, J. L., Proc. Soc. Exp. Biol. and Med., 1964, v116, 1131.

3. Pope, J. H., Rowe, W. P., J. Exp. Med., 1964, v120, 121.

4. Kitahara, T., Butel, J. S., Rapp, F., Melnick, J. L., Nature, 1965, in press.

5. Rapp, F., J. Bact., 1964, v88, 1448.

6. Rapp, F., Hsu, T. C., Virology, 1965, in press.

7. Rapp, F., Vanderslice, D., ibid., 1964, v22, 321.

8. Rapp, F., Kitahara, T., Butel, J. S., Melnick, J. L., Proc. Nat. Acad. Sci., U. S., 1964, v52, 1138.

9. Rapp, F., J. Immunol., 1962, v88, 732.

10. Girardi, A. J., Hilleman, M. R., Zwickey, R. E., Proc. Soc. Exp. Biol. and Med., 1962, v111, 84.

11. Moore, A. E., Caparo, A. C., Cancer Res., 1964, v24, 765.

12. Girardi, A. J., Hilleman, M. R., Zwickey, R. E., Proc. Soc. Exp. Biol. and Med., 1963, v114, 609.

13. Girardi, A. J., Slotnick, V. B., Hilleman, M. R., ibid., 1962, v110, 776.

14. Sabin, A. B., Koch, M. A., Proc. Nat. Acad. Sci., U. S., 1964, v52, 1131.

15. Huebner, R. J., Rowe, W. P., Turner, H. C., Lane, W. T., ibid., 1963, v50, 379.

Received November 16, 1964. P.S.E.B.M., 1965, v118.

Immunofluorescence of Newcastle Disease Virus in Paraffin Embedded Tissues of Early Chick Embryos.* (29908)

ALICE POLK WILLIAMSON AND RUSSELL J. BLATTNER
Department of Pediatrics, Baylor University College of Medicine, Houston, Texas

Three members of the myxovirus group (mumps, influenza A, and Newcastle disease virus) have been studied previously in early chick embryos and each was observed to produce developmental defects, differing somewhat for each of the viruses(1-6). Essentially, however, the defects occurred in tissues which were in early stages of differentiation and which were directly exposed to the inoculated virus. Although increase in virus titer was observed in all 3 instances it was not known whether the defects were caused by selective replication of virus in the tissues affected or whether the virus replicated in all cells but caused damage only in certain tissues which were vulnerable because of a particularly sensitive phase in their developmental process. To study the distribution of the replicating virus in the early chick embryo, a method was necessary which would allow identification of the virus in serially sectioned embryos. The successful use of a paraffin embedding technique for immunofluorescent staining has been reported(7) by Guy Ste.-Marie using several types of antigen including influenza A virus. These techniques have been adapted for staining Newcastle disease virus in serially sectioned tissues of the early chick embryo.

* Supported by PHS Grant HD 00298 from Nat. Inst. of Child Health and Human Development.

Methods and materials. Preparation of the conjugate. Viral antigen to be used for antibody production was grown in the allantoic cavity of the chick embryo. Young adult chickens were immunized by intranasal instillation of the Blacksburg strain of Newcastle disease virus (NDV), and this was followed in about 5 weeks by intramuscular injections of 0.4 ml of the Roakin strain of NDV of ID_{50} $10^{10.9}$ (2 injections 3 days apart). Ten to twelve days after the final injection, the chickens were bled. Hemagglutination-inhibition titers of the sera ranged between 1-2000 and 1-4000. The globulins were extracted and conjugated to fluorescein isothiocyanate by the method of Coons(8) modified by Riggs *et al*(9). Instead of the dialysis techniques to remove the excess dye, the conjugate was cleared by passing through

a Sephadex column(10). It was then dispensed in ampules, hermetically sealed and stored in the frozen state at —10°C. Just before use the nonspecific antibodies were removed by absorbing twice with chick embryo powder prepared from 8-day and 3-day-old chick embryos mixed in the proportion of 6 to 1 by dry weight. Such absorbed conjugates were always used within 2 or 3 days of preparation. For staining, the conjugate was appropriately diluted, usually 1-12, in saline buffered at pH 8.8. New preparations of conjugate could be titrated for optimum staining-dilution by staining smears of NDV-red cell mixtures fixed in 95% ethanol at 2°C.

Preparation of embryos. Methods of inoculating and harvesting the young embryos have been previously described(1,2). Chick embryos of 48 hours incubation, inoculated with allantoic fluid suspensions of the Roakin strain of NDV of an ID_{50} titer of $10^{5.3}$, were harvested between 16 and 24 hours after inoculation. The embryos were floated briefly in saline where the vitelline membranes were removed. They were then immersed immediately in 95% ethyl alcohol at 2°C and processed for paraffin embedding techniques as described by Ste.-Marie(7). Serial sections were cut of the entire embryo, and the ribbons were laid out in order in shallow boxes lined with black paper. Gross structures such as brain, eye, visceral arches, heart, auditory vesicles, etc., could be identified fairly accurately by observing the paraffin ribbons at 10 × magnification.

Preparation and examination of the stained slides. To conserve time and stain, specific areas were selected for staining in these initial studies. Approximately 12 to 16 adjacent sections were mounted on the slides in such a way that the sections on the 2 halves of the slide could be separated by a line made with an ether-vaseline mixture. The two sides could then be subjected to different staining procedures when necessary for control purposes. The boxes containing the rest of the sections were stored in the dark at 4°C, and other areas were stained as needed. Deparaffinization of the slides was done at room temperature in 3 consecutive xylene baths of 1 minute each followed by 1 minute each in ethanol 95%, 80%, 60% and 40%, and finally 2 changes of saline (pH 7.2) for 10 minutes each. The slides were then stained with diluted conjugate for 30 minutes in a moist chamber. They were then washed 25 minutes by gentle agitation in saline (pH 8.8) kept in an ice bath. At this point the stained slides were fixed in 95% ethanol for 15 minutes at 2°C followed by briefly washing 3 times in the staining buffer. The fluorescence in the slides was thus preserved for several days. Without this final fixation the fluorescence faded rapidly so that the slides had to be examined immediately after staining. The stained sections were examined under a Carl Zeiss fluorescence microscope using BG 12 exciter filter and a yellow barrier filter. After examination the coverslips were soaked off in water, and the sections were restained with hematoxylin and eosin for examination under the conventional microscope.

Results. Specificity of the staining reaction. Sections of 7 embryos from eggs inoculated with NDV 16 to 24 hours previously all showed bright fluorescence distributed widely in areas of the extraembryonic membranes and in certain tissues of the embryo proper (Figs. 1-3). Adjacent sections stained with conjugate from unimmunized chickens failed to show a similar fluorescence. Furthermore fluorescence was inhibited in sections which had been pretreated with unconjugated immune globulin prior to staining with specific conjugate. Uninoculated control embryos failed to show the specific staining reaction with immune conjugate. The color of the fluorescence was a characteristic bright green. The only other areas where a similar color was seen were the yolk globules. This staining characteristic of the yolk was nonspecific, since it appeared in both control and experimental sections. Vitelline membrane, if it had not been removed, also stained a brilliant green and interfered with the study of the specific staining reaction. Red blood cells of the chick embryo were normally brighter than other tissues, but in well stained sections this color was slightly pinkish in contrast to the clear bright green of the viral antibody.

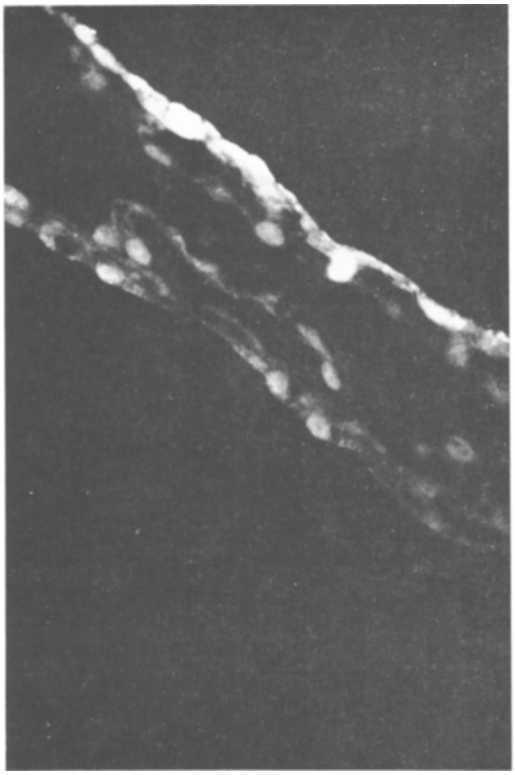

FIG. 1. Virus in ectodermal cells of chorion of chick embryo inoculated with NDV at 48 hr incubation and harvested 24 hr later. Immunofluorescent staining of NDV. ×400.

Distribution of the virus in the tissues. The viral antigen was located in many cells of the developing embryo, sometimes at the cell wall and sometimes within the cytoplasm but not in the nucleus of the cells. The areas of fluorescence corresponded in general with the areas where gross defects or characteristic microscopic pathological changes had previously been observed(3,11), *i.e.*, in tissues differentiating from the body ectoderm including the lens primordia, auditory vesicles, visceral arch ectoderm, olfactory pits, and it was also found in visceral arch mesoderm and in the epimyocardium of the heart. Virus was frequently found also in body ectoderm immediately adjacent to these structures. In areas where mesenchyme lay immediately beneath the affected area, fluorescent cells were scattered throughout the superficial areas of the mesenchyme. Cells of the body ectoderm just lateral to the dorsal midline appeared also to be unusually susceptible to infection by the virus. Although more fluorescent cells were observed in the organs described, the fluorescence was not limited to these areas but was also observed in cells of the general body ectoderm. In embryos infected the longest, increasingly more of the cells of the general body ectoderm were affected and more extensive invasion of the mesenchyme was observed. Toward the caudal end of the embryo the cells most consistently affected were the ectodermal cells at the lateral limiting sulcus. In some embryos ectoderm over somites was affected. In younger embryos, the unclosed portion of the neural tube has previously been observed to be susceptible to tissue damage following NDV infection. In embryos of the present study the neural tubes had closed, but in a few embryos fluorescence was observed in the neural tube, generally at the dorsal portion and in the overlying ectoderm. Little tissue damage was seen in

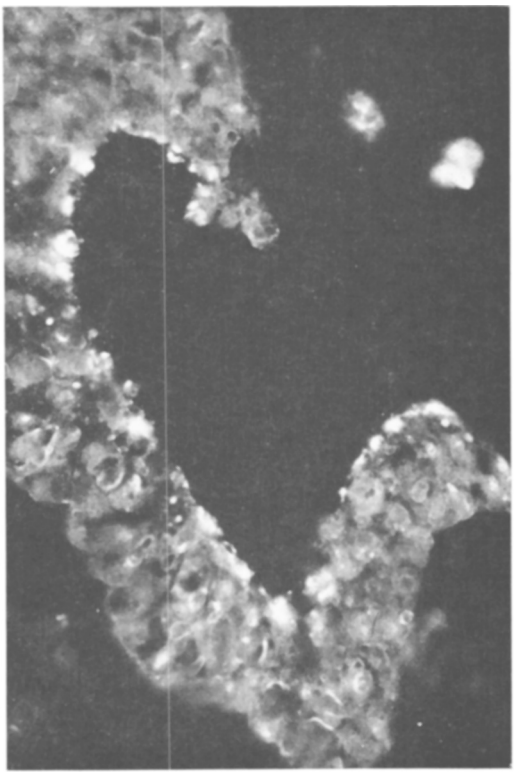

FIG. 2. Virus in surface cells of the auditory vesicle of chick embryo inoculated with NDV at 48 hr incubation and harvested 24 hr later. Immunofluorescent staining of NDV. ×400.

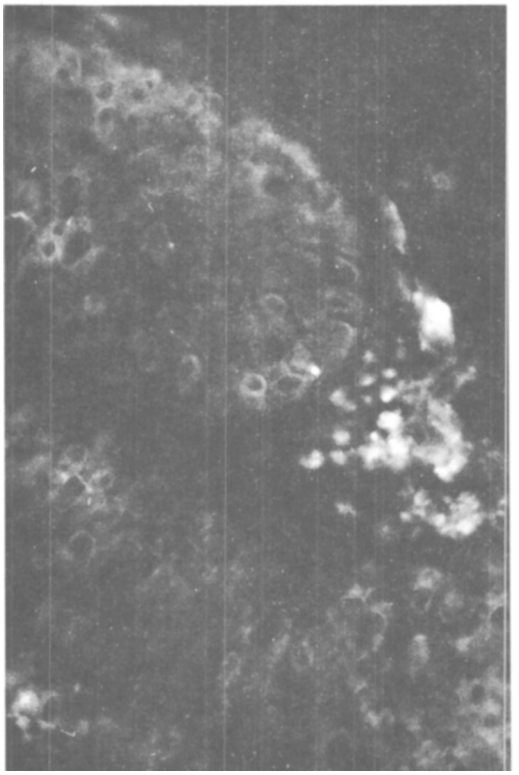

FIG. 3. Virus in degenerating cells of the lens vesicle of chick embryo inoculated with NDV at 48 hr incubation and harvested 24 hr later. Immunofluorescent staining of NDV. ×400.

the general body ectoderm. In contrast, however, sloughing of virus infected cells and extensive cell degeneration was observed in virus infected organs which were differentiating from the body ectoderm (Fig. 2, 3).

Virus was also observed to be widely distributed in cells of the extraembryonic membranes, *i.e.*, the chorion, amnion and yolk sac entoderm. The ectodermal layers were primarily affected in the chorion and amnion but extension to the underlying mesoderm was not infrequent. In the extraembryonic membranes, fluorescence was consistently observed to be brightest and most concentrated in the ectamnion (closing border of the amnion). Thus, in these early chick embryos, susceptibility to NDV was found among cells derived from all three primary germ layers, ectoderm, mesoderm and entoderm. Little tissue damage was observed in the extraembryonic membranes except for cell degeneration in the affected ectamnion and in small islands of hyperplasia which were occasionally observed in the chorion at the site of fluorescence.

The localization of NDV in early chick embryos by these techniques has made it possible to use serial sections for studying the fundamental relationship of NDV replication to the characteristic developmental changes observed. Further experiments are in progress to determine in which tissues the virus first appears and to compare the distribution of virus in embryos inoculated at different stages of development.

Summary. Paraffin embedded tissues have been found to be suitable for immunofluorescent studies of Newcastle disease virus-induced defects in chick embryos inoculated at early stages of incubation. In embryos inoculated at 48 hours incubation and harvested 16 to 24 hours later viral antigen was observed in many tissues of the embryo proper and in cells of the extraembryonic membranes. The viral antigen was most concentrated in the organs subject to the virus-induced developmental defects, *i.e.*, in tissues differentiating from the body ectoderm, including lens primordia, auditory vesicles, visceral arch ectoderm, olfactory epithelium and it was also found in the visceral arch mesoderm and in the epimyocardium of the heart. However, virus was not restricted to these tissues but was also observed in some cells of the general body ectoderm and was widely distributed in cells of the chorion, amnion, and in the yolk sac entoderm. In late infections the specific viral fluorescence was also seen in mesodermal tissues directly underlying the affected ectoderm. Thus viral antigen was found to be distributed in cells derived from all three of the primary germ layers, ectoderm, entoderm, and mesoderm.

1. Blattner, R. J., Williamson, A. P., Proc. Soc. Exp. Biol. and Med., 1951, v77, 619.

2. Williamson, A. P., Blattner, R. J., Robertson, G. G., J. Immunol., 1953, v71, 207.

3. Robertson, G. G., Williamson, A. P., Blattner, R. J., J. Exp. Zool., 1955, v129, 5.

4. Williamson, A. P., Blattner, R. J., Simonsen, L., J. Immunol., 1956, v76, 275.

5. Williamson, A. P., Simonsen, L., Blattner, R. J., Proc. Soc. Exp. Biol. & Med., 1956, v92, 334.

6. Williamson, A. P., Blattner, R. J., Simonsen, L., ibid., 1957, v96, 224.

7. Sainte-Marie, G., J. Histochem. & Cytochem., 1962, v10, 250.

8. Coons, A. H., Kaplan, M. H., J. Exp. Med., 1950, v91, 1.

9. Riggs, J. L., Seiwald, R. J., Burkhalter, J. H., Downs, C. M., Metcalf, T. G., Am. J. Path., 1958, v34, 1081.

10. Pharmacia, Ltd., Brochure on Sephadex, Uppsala, 1959.

11. Robertson, G. G., Williamson, A. P., Blattner, R. J., Simonsen, L., Anat. Rec., 1959, v133, 455.

Received November 18, 1964. P.S.E.B.M., 1965, v118.

Gastric Cannula: A Modification to Combat Leakage.* (29909)

MICHAEL D. MIDDLETON† AND LLOYD M. NYHUS (Introduced by H. N. Harkins)
Department of Surgery, University of Washington School of Medicine, Seattle

Long term animal experiments, which involve the collection of small quantities of secretions from gastric pouches, are often seriously upset by the development of a leakage of secretions around the cannula. Experiments designed to compare pouch acid output after various consecutive procedures are valueless if a leak develops around the cannula. In these circumstances, reimplantation of the cannula may so alter the secretory capacity of the pouch as to negate all further observations.

The major problem of preventing complete erosion of the cannula through the pouch has for all practical purposes been solved by constructing the pouch in the manner used in this laboratory(1) and by employing the improved cannula described by Condon and Harkins(2).

However, a certain proportion of Heidenhain pouches so constructed develop leakage around the cannula after several months' experimentation. This is especially noticeable when the method of collection is that described by Savage, Stavney, Nyhus and Harkins(3), a method which strains the leakproof qualities of any cannula.

This problem has caused us considerable difficulty, and we have devised a simple addition to the cannula described by Condon and Harkins(2), which has considerably reduced the incidence of this problem. This addition consists of a Silastic‡ rubber ring which fits securely around the gastric end of the cannula. Its outer surface is beveled as shown in the diagram. The measurements given are those which will fit our cannula (Fig. 1). Addition of this Silastic ring gives the gastric end of the cannula a "sloping shoulder" (Fig. 2). The cannula is inserted in the manner previously described from this laboratory(1).

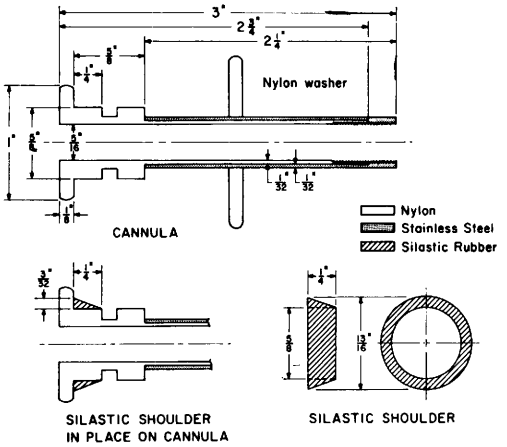

FIG. 1. Mechanical drawing of cannula and Silastic shoulder.

Several months' use of this modification in many dogs has convinced us of its efficacy. If after some months the cannula becomes loose and can be easily rotated, the slight weight of any attached collecting apparatus is sufficient to pull the cannula down and force the sloped shoulders into the orifice of

* Supported by USPHS Grant #AM-04010 from Nat. Inst. Health.

† Visiting Scientist, formerly Senior Registrar, Radcliffe Infirmary, Oxford, England.

‡ Medical grade Silicone rubber (Dow Corning).