rodents, androgens had an augmenting action on erythropoiesis(10). This action required the presence of the kidneys, suggesting a renal mechanism, although direct action of testosterone on erythrogenic tissue grown in tissue culture has been reported(13). It is possible that a lack of an enhancing effect by androgens on erythropoiesis in Friend virus-infected mice may be due to a dose response.

Summary. The effect of pituitary and gonadal hormones on Friend Virus Disease in mice has been studied. Estradiol inhibited the virus-induced hypervolemic polycythemia, hepatomegaly, and splenomegaly. The effect was most evident during the second week following injection. Progesterone, adrenocorticotropic hormone, and testosterone did not influence significantly the hematologic and organ changes characterizing the disease. A possible mechanism of action of estrogen-induced suppressions of erythropoiesis in this disease is discussed.

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An Immunofluorescent and Histopathological Study of Respiratory Syncytial (RS) Virus Encephalitis in Suckling Mice.*† (31923)

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In our initial investigation of the adaptation of the Long strain of respiratory syncytial virus(1) to newborn mice, we reported that the mouse-passed RS virus could not be passed intracerebrally in mice 3 to 4 weeks of age(2). Subsequently, the susceptibility of suckling mice of different ages was examined periodically during the serial intracerebral passages. The present investigation indicates that the neuropathic strain of RS virus may be propagated in 7- and 9-day-old mice, and describes the application of histologic, virus assay and direct immunofluorescent staining techniques to the study of the pathogenesis of the infection produced in suckling mice by the neuropathic strain of RS virus.

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Materials and methods. Virus. The neuropathic strain of RS virus (RMB-RS) derived in this laboratory, was used throughout this study. The stock virus was prepared and stored as a 20% suspension of infected mouse brain as described previously(2). Details of the procedures used for growth and maintenance of tissue culture (TC) and for virus assay *in vitro* and *in vivo* (mice) have been published(2).

Preparation of the conjugates. RS virus immune horse serum, which had been prepared against the Long strain of RS virus, was purchased from Flow Laboratories, Inc., Rockville, Md., with a neutralization titer of 1:640. The conjugation procedure was done at 4°C. The immune and normal horse sera were fractionated by 50% saturation with ammonium sulfate. The globulin solutions were conjugated with fluorescein isothiocyanate(3) and the conjugates fractionated by the cellulose anion exchange method.

Specificity of conjugated antiserum to RS virus. The specificity of the conjugated serum was determined by preliminary staining of uninfected HL cells and HL cells infected with the Long strain of RS virus. Fluorescence was not observed when coverslips at various stages of infection were treated with conjugated normal horse serum. Likewise, fluorescence was not observed when noninfected coverslips of HL cells were treated with labelled immune serum. In addition, fluorescence was inhibited when HL cells infected with RS virus were treated with labelled immune RS serum which had been diluted in homologous unlabelled RS virus immune serum.

Preparation of infected tissue for examination. The tissues for immunofluorescent and histological studies were promptly removed after the sacrifice of the animal and placed immediately into 95% ethanol, pre-cooled at 4°C, and processed for paraffin embedding as described by Sainte-Marie(5).

Serial sectioning of all the tissues was performed at 5 mμ with a rotary microtome with flotation of the tissue ribbons on warm water (40°C).

Staining procedures for infected tissues. Prior to immunofluorescent staining, the tissue sections were deparaffinized at room temperature by passing the slides through 2 consecutive baths of xylene for a total of 10-15 minutes; this was followed by one minute each in absolute ethanol and 95%, 80%, and 50% ethanol with final passage through 3 changes of phosphate buffered saline (pH 7.4).

Fluorescent antibody staining was done by the direct method(6). Slides were overlaid with the conjugated sera in the following manner: one of the two adjacent sections on the slide was overlaid with anti-RS conjugate diluted 1:2 or 1:4 in phosphate buffered saline; as a control, the other section was stained with either anti-RS immune serum or unlabelled normal serum. The slides were then incubated for 35 minutes at 37°C and washed 3 times (room temperature) in phosphate buffered saline (pH 7.4) for two 10-minute intervals, after which coverslips were mounted in buffered glycerol. Following examination for specific fluorescence, the coverslips were floated off in PBS and the sections were restained with hematoxylin and eosin.

Microscopy. A Zeiss microscope, equipped for fluorescence observations and illuminated with a Osram HBO 200 lamp was used for the fluorescence microscopy. The filter system consisted of a UG 5 exciter filter and an OG 4-GG4 barrier filter. The hematoxylin and eosin sections were examined by conventional means.

Results. Gradual increase in age of suckling mice susceptible to neuropathic RS virus. Our early studies had shown that the mouse-passed RS virus could not be passed intracerebrally in 3-4-week-old mice(2). However, beginnning with the one-day-old mouse, the adaptation of the virus to suckling mice of increasing ages was examined periodically during serial intracerebral passages. At each age level the initial passage of the virus was not lethal, but on subsequent serial passages, usually 5 intracerebral passages, pathological manifestations as described in the text were evident, followed by death. The neuropathic strain of RS virus was also adapted to grow in the brains of 7- and 9-day-old mice with no attempt made to proceed further with infection of older or adult mice. The neuropathic

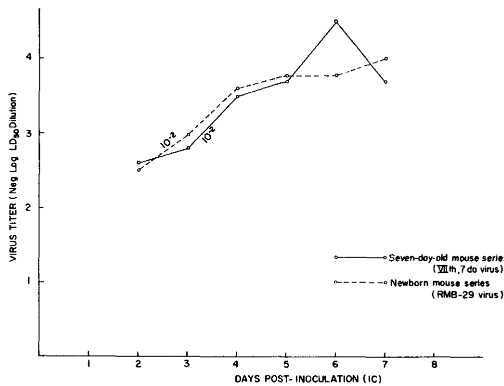

FIG. 1. Growth curves of neuropathic strain of RS virus in mice inoculated at 7 days of age and at less than 24 hr of age.

strain of RS virus has been carried through 11 passages in 7-day-old mice and 15 passages in 9-day-old mice. The LD_{50} titers in the 7-day-old mice have ranged between $10^{3.2}$ and $10^{4.0}$ LD_{50} per 0.03 ml; titers in the 9-day-old have averaged $10^{2.5}$ LD_{50} per 0.03 ml.

Growth of neuropathic RS virus in mouse brain. Fig. 1 presents the growth curves of neuropathic RS virus in mice inoculated intracerebrally both at 7 days of age and at less than 24 hours of age and depicts the progressive rise in the infectivity titer of the virus. Each value represents the titer of infectious virus from brains of 3 mice. No striking differences were noted between the two curves.

Pathogenesis of neuropathic RS virus infection. The course of the mouse-brain adapted RS virus infection in intracerebrally inoculated 7-day-old mice was followed daily by means of fluorescent antibody and hematoxylin and eosin staining. The observations were correlated with the growth cycle of the virus in these animals.

Seven-day-old mice were inoculated intracerebrally with 0.03 ml of 10^{-2} dilution of brain suspension of neuropathic RS virus with an initial infectivity titer of $10^{3.5}$ LD_{50} per 0.03 ml. On day 2 post-inoculation and daily thereafter, 3 mice were sacrificed and from each animal the brain, lungs, liver and kidneys removed and prepared for immunofluorescent and histopathological examination.

Clinical features. The clinical signs of RS virus infection in these mice appeared 3 to 5 days post-inoculation. The early stages of the infection were characterized by hyperexcitability. Later, the diseased animals became lethargic and ataxia and tremors could be observed. In some cases, convulsions occurred spontaneously or could be induced by twirling the mice by their tails. Deaths generally occurred one to three days after the appearance of the clinical signs.

Histological and immunofluorescent findings. As was suggested by the clinical signs of illness, the infection was found to be limited to the brain. Conventional histopathological examination of lung, liver, heart, and kidney of the intracerebrally inoculated mice revealed no detectable lesions. In addition, fluorescent-antibody staining did not show RS antigen in these tissues. These observations were complemented by attempts to isolate RS virus from these tissues. Suspensions prepared from the tissues and assayed in HL cell cultures for the presence of RS virus yielded negative results. In contrast, the virus could always be recovered from the brains of the inoculated mice.

In general, definite microscopic cerebral lesions first became manifest on the third day after intracerebral inoculation of the virus and progressed in severity until the death of the mice, at 6 to 8 days post-inoculation. This pattern paralleled the growth cycle of the virus (Fig. 1) in that the maximum infectivity titer occurred on days 6 to 8 post-inoculation.

In mice sacrificed 2 days after intracerebral inoculation, no areas of necrosis or inflammatory infiltrate were noted within the substance of the brain. Immunofluorescent staining of these sections revealed only a small number of cells showing specific fluorescence of low intensity. On the third and fourth days post-inoculation, small foci of early necrosis were observed in the cortex. In these areas, there were dying neurons, nuclear debris, a few polymorphonuclear cells and microglial cells. The affected neurons exhibited pyknotic or fragmented nuclei; their cytoplasm was shrunken and deeply eosinophilic. There was evidence of beginning liquefaction of the necrotic areas (Fig. 2). At this time, the number of fluorescent cells and the intensity of their fluorescence was found to have increased. The fluorescence appeared to be

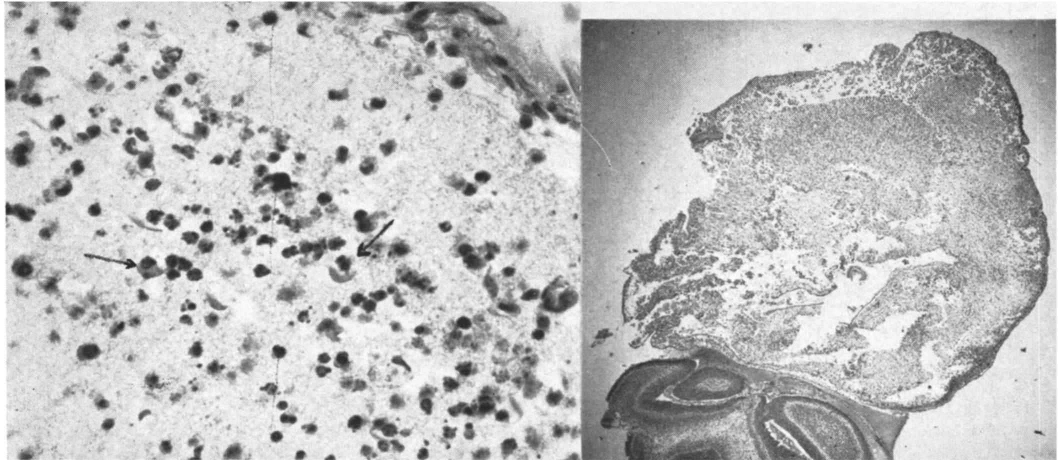

FIG. 2. High power view of area of early necrosis. Degenerating neurons (arrows) are recognized by the slightly pyknotic nuclei and shrunken cytoplasm. Frankly necrotic cells show disintegration of their cytoplasm and nuclei, liquefaction just beginning. Hematoxylin and eosin. ×620.

FIG. 3. Brain of mouse inoculated intracerebrally at less than 24 hours of age with neuropathic RS virus and sacrificed at 7 days post-inoculation. Extensive destruction is evident, with liquefaction of the major portions of the cerebrum. Hematoxylin and eosin. ×32.

concentrated in cells of areas showing early lesions histologically.

The mice sacrificed on days 5 through 8 showed a progressive increase in the severity and extent of cerebral destruction.

On day 5, liquefaction of the damaged areas was well marked. The lesions contained pyknotic or fragmented nuclear debris, and neurons in varying stages of necrosis. There was increased accumulation of microglial cells and advanced lesions contained many large vacuolated microglial cells *(Gitterzellen)*. The appearance of swollen astrocytes was first noted at this stage; these were seen at the margins of areas of destruction.

As the infection progressed, the foci of involvement became more numerous and the areas of liquefaction increased in size. There was in the late stages, days 6 to 8, complete destruction of major portions of the cerebrum (Fig. 3).

Specific fluorescent staining became progressively more widespread, corresponding well within the areas of histologically demonstrable lesions. Individual fluorescent cells were difficult to identify specifically as to cell type, largely due to the limitations of immunofluorescent observations (Fig. 4). As judged from hematoxylin-eosin staining of the same sections, which had been subjected to immunofluorescent examination, many of the fluorescent cells appeared to be neurons. The possibility that some of the fluorescent cells were glial or phagocytic can not be ruled out. All the specific fluorescence was intracytoplasmic; no nuclear fluorescence was observed. Similar histopathological changes were seen upon infection of newborn mice (less than 24 hours old) with the neuropathic strain of RS virus.

Extracerebral inoculation of mice with the neuropathic strain. Throughout serial intracerebral passage in newborn and 7-day-old mice, the neuropathic strain was tested at periodic intervals for its ability to infect mice by routes other than the intracerebral, such as intranasal, intraperitoneal or intrathoracic. It was not possible to initiate serial passages by these routes, nor was there evidence of any neurological involvement, nor were we able to recover the virus from the brain of mice.

Discussion. The adapted strain of RS virus appears to be strickly neuropathic, in the sense that it produces lesions only in the nervous system of intracerebrally inoculated suckling mice and not in any other organs of the animal. Furthermore, cerebral infection or neurological symptoms were not produced by extracerebral inoculation of the virus, and neither thoracic nor abdominal organs were

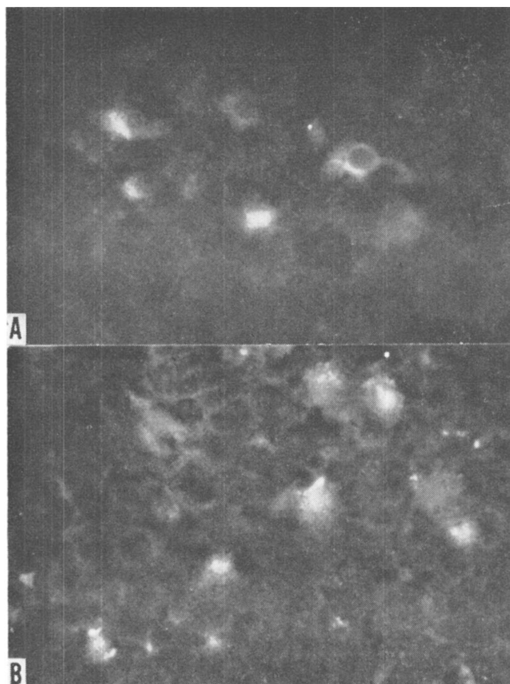

FIG. 4. Cerebral cortex of mouse inoculated intracerebrally with neuropathic RS virus.
a. Sacrificed at 5 days post-inoculation
b. Sacrificed at 8 days post-inoculation
Immunofluorescent staining of RS virus antigen in cytoplasm of host cells. ×640.

involved. The results correlate quite well with the infectivity determinations, as the extracerebral tissues tested were completely negative. These observations are similar to those noted by Francis and Moore(7) with the NWS strain of influenza. They observed that pulmonary lesions were not seen in mice injected intracerebrally with NWS virus and neurological signs were not observed in mice receiving neurotropic NWS intranasally.

The pathologic lesions induced by the virus consisted of death of the cells without characteristic syncytial or giant-cell formations. The lesions appeared to be similar to those produced by other murine adapted strains of myxoviruses (subgroup II), such as measles, canine distemper and rinderpest viruses(8). The most striking feature was the destruction of the neurons, in both the grey and white matter.

In all experiments, the cerebral fluorescent staining was intracytoplasmic; no specific nuclear fluorescent staining was observed. This finding agrees with the immunofluorescent observations of cell cultures infected with RS virus(9). The distribution and amount of the pathological and immunofluorescent lesions was less widespread in mice inoculated at 7 days of age than in those inoculated within 24 hours after birth. This may be explained on the basis that in older mice there was an increase in resistance to RS virus with increasing age resulting in a reduction of the number of cells infected. This explanation appears to complement well the observation that, in older mice, although fewer cells were seen with specific fluorescence, these were of undiminished fluorescent intensity.

The increase on successive days of infection in the amount, intensity, and distribution of the specifically fluorescent cells paralleled the progressive increase in the infectivity titer of the virus. Failure to observe intense specific fluorescence prior to days 3 or 4 post-inoculation reflects the low infectivity titer of the virus at that stage of its growth cycle in mouse brain. It is suggested that prior to days 3 or 4 post-inoculation the amount of infective virus associated with the RS virus infection was too low to be detected by fluorescent-antibody staining. The efficacy of this mouse-adapted RS virus host system suggests practical applications in assaying and evaluating biological and/or chemical agents useful in combating infection of man with the respiratory syncytial virus.

Summary. The susceptibility of suckling mice of different ages to the neuropathic strain of respiratory syncytial (RS) virus was examined periodically during serial intracerebral passage(s) of the virus in newborn mice. By passing the virus serially in mouse brain, beginning with the one-day-old mouse and gradually increasing the age, it became possible to grow the RS virus in 7- and 9-day-old mice. The pathogenesis of the encephalitis produced by neuropathic strain of the RS virus, in intracerebrally inoculated suckling mice, was studied by means of immunofluorescent and histopathologic techniques. The histological changes were correlated with immunofluorescence and infectivity titrations. The clinical evidence of encephalitis in the infected mice was found to be associated with

extensive necrosis and liquefaction of the brain. This mouse brain adapted strain of RS virus was found to be strictly neuropathic, in that it produced lesions only in the nervous system of the intracerebrally inoculated suckling mice and failed to produce either infection or lesions in any of the extracerebral tissues which were examined.

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An Enhancing Factor in Normal Chicken Serum Augmenting Saline Agglutination of a Mycoplasma Antigen-Antibody System.* (31924)

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During experiments with agglutination tests involving antisera against *Mycoplasma gallisepticum* (MG), it was noted that the addition of serum from apparently normal chickens augmented the saline agglutination by 2-3 doubling dilutions, whereas serum from turkeys had no effect. Saline and antiglobulin determinations on these normal chicken sera failed to demonstrate antibodies directed against MG.

A review of the literature failed to reveal previous studies of a factor in normal chicken serum that would enhance agglutination. However, Makinodan *et al*(5) had demonstrated a macroglobulin in normal chicken serum that coprecipitated with antibody against bovine serum albumin (BSA). This coprecipitation occurred with aged normal serum or in the presence of 1.5 M NaCl, whether the serum was aged or not. Similarly, Orlans *et al* (6) showed that addition of normal fowl serum increased the amount of specific precipitate with anti-BSA serum. The yields of precipitate were greater with 0.9% NaCl than with 8% NaCl. Subsequently, Orlans and Rose(7) determined that the increased precipitation attributed to normal serum was due to one or more factors, including a macroglobulin. These factors were heat-stable (56° for 30 minutes) but were destroyed at pH 5.5 or lower and were inhibited with ethylenediaminetetraacetic acid (EDTA). The present communication deals with some of the characteristics of agglutinating enhancing factor (EF).

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Experimental. Antigens. The antigens employed were as follows: 1) *M. gallisepticum* prepared by a procedure described previously (1); 2) similarly prepared antigens from a nonpathogenic avian mycoplasma strain designated simply as Tu; 3) *Salmonella typhimurium* "O" antigen prepared by a standard method(4); 4) *Haemophilus gallinarum* antigen (obtained from Dr. Richard Yamamoto of this Department); and 5) washed avian red blood cells collected from Single-Comb White Leghorn chickens (SCWL).

Antisera. MG antisera were prepared in chickens and turkeys; the other antisera used were of chicken origin. Antibodies directed against MG were prepared by nasal or tracheal infection of chickens and turkeys with live organisms, whereas Tu antisera were raised by the intravenous inoculation of live