the hypothesis that interference with glycolysis would inhibit neoplasia.

Combinations of glyceraldehyde, azaserine, and chloropurine were more effective than once daily treatments with glyceraldehyde or with azaserine and chloropurine. The results obtained were comparable to those observed using the same doses of azaserine and chloropurine given twice daily(4). The ability to demonstrate therapeutic synergy in the present system was limited by the potency of azaserine and chloropurine.

Summary. DL-glyceraldehyde prolonged survival time of mice bearing Sarcoma 180 or Hepatoma 134 ascites cells. Mecca lymphosarcoma ascites cells proved resistant to this agent. Combination of glyceraldehyde, azaserine, and chloropurine in the therapy of Sarcoma 180 produced a greater effect than that found using glyceraldehyde alone or with the dose level of azaserine and chloropurine employed.

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Site of Intracellular Antigen Production by Myxoviruses.* (25803)

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Application of fluorescent antibody technics to the study of multiplication of myxoviruses has suggested that the nucleoprotein component of influenza(1,2) and fowl plague(3, 4) viruses is produced within the nuclei of infected cells. The most complete study of this phenomenon is that by Breitenfeld and Schafer(3). From their experiments with chicken embryo fibroblasts infected with fowl plague virus they concluded that viral hemagglutinin is produced in the cytoplasm, whereas the nucleoprotein component of the virus particle is produced within the nucleus and later migrates to the periphery of the cell where it is combined with hemagglutinin to form complete, infectious virus particles. In our studies of the effect of Sendai virus on cells it was found that intranuclear antigen was not demonstrable by use of fluorescent antibody. Since this appeared to represent an important difference in characteristics of multiplication of viruses within the myxovirus group, the phenomenon was studied further. This paper compares the multiplication cycles of Sendai, mumps, and Newcastle disease viruses with that of influenza A virus in regard to intracellular site of antigen production.

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Materials and methods. Viruses. The PR-8 strain of influenza A, the Roakin strain of Newcastle disease virus (NDV), and the Enders strain of mumps virus were used. These were egg-adapted lines of virus cultivated by standard methods in the allantoic sac of chicken embryos. The Dunai strain of mumps virus(5) adapted to serial cultivation in human conjunctiva cells was used as virus from the 8th tissue culture passage. The Sendai strain of para-influenza I virus was cultivated in the allantoic sac of chicken embryos(5). A line of Sendai virus adapted to serial cultivation in human conjunctiva cells was used as virus from the 7th tissue culture passage. *Cell cultures.* Primary cultures of chick embryo lung cells were prepared from lungs of 11-day-old embryos(6). They were grown in Scherer's medium with addition of 5% chick embryo extract and 15% horse serum and were maintained in this medium after inoculation. Human conjunctiva cells (Chang) were grown in serial culture in Eagle's basal medium containing 20% horse serum and after inoculation

were maintained in Scherer's medium containing 5% horse serum. For examination by fluorescence microscopy cells were grown on cover-glasses in tubes with flattened sides. Before inoculation the medium was removed and the cells washed with a balanced salt solution. Cells were exposed to the inoculum, containing the appropriate quantity of virus in 1.0 ml of culture maintenance medium, for 30 min. at room temperature, after which the inoculum was removed, the cell sheet was washed with balanced saline, and 1.0 ml of maintenance medium added. Timing of virus growth cycles was begun 15 min after addition of virus inoculum. At selected intervals the medium was removed from cultures, and the cells were washed twice with balanced saline. Cover-glasses were removed, fixed in acetone for 10 min, then dried in air. Cell preparations were stored in a desiccator at 4°C until time of microscopic study. *Antisera.* To produce antiserum against influenza and Sendai viruses mice were given an intranasal instillation of diluted infected allantoic fluid. An inoculum was selected that resulted in pulmonary infection and about 10% mortality. Three weeks after inoculation the mice were given an intraperitoneal injection of 0.05 ml of a saline extract of 20% infected mouse lung homogenate. This was repeated one week later and the mice were exsanguinated 1 week after the second booster injection. Antisera were produced against Sendai and mumps viruses in 800 g guinea pigs by giving the animals an intranasal instillation of 0.5 ml of undiluted infected allantoic fluid. Guinea pigs given mumps virus were bled 2 weeks after inoculation and those given Sendai virus were bled 3 weeks after inoculation. Antiserum against NDV was produced by infecting adult chickens *via* the intranasal route with an inoculum that caused obvious illness in most chickens and an occasional death. Chickens were bled 3 weeks after inoculation. Except for chicken sera, all sera were tested by complement-fixation for antibody content against the S (nucleoprotein) and V (hemagglutinin protein) antigens of the appropriate virus. Sera selected for conjugation with fluorescein were ones with good levels of antibody against both viral antigens. Gamma globulin was precipitated from antisera with methanol and conjugated with fluorescein isothiocyanate (7). Conjugated preparations were absorbed with mouse liver powder and chick embryo powder. *Microscopy.* Infected cells on cover slips were stained with fluorescent antibody using the direct method(8). Controls consisted of uninfected cells treated with specific conjugate, infected cells treated with normal serum, and, on certain occasions, infected cells pre-treated with unlabeled antibody prior to staining with labeled antibody. Slides were examined using a 200 watt high-pressure mercury-vapor lamp UV light source and a BG 12 excitor filter. For observation a Wratten 3 barrier filter was used. Photographs were made using a Wratten K2 barrier filter, Kodak Plus-X film, and an exposure time of 2 min.

Results. Influenza virus. Watson and Coons(1) noted a nuclear phase in multiplication of influenza virus in the chicken embryo and similar observations were made by Liu (2) who studied the infected respiratory epithelium of ferrets. No detailed study employing fluorescent antibody and following development of the PR8 strain of influenza A virus in a single cycle of multiplication had been published, however, so it was necessary to examine the cycle of influenza virus to be able to compare it with cycles of NDV, mumps and Sendai viruses.

Cultures of chicken embryo lung cells were inoculated with the PR8 strain of influenza A virus using a multiplicity of approximately 10 egg-infectivity doses (EID_{50}) per cell. Observations were made at 1, 2, 4, 6, 8, 12, 20, and 26 hr after inoculation. No specific fluorescence was noted in the cells until 6 hr, at which time bright yellow granules were seen in the nuclei of some cells. The nuclear membrane of such cells appeared as a fine yellow line; no specific fluorescence was noted in the cytoplasm. At 8 hr in most cells the nucleus fluoresced as a solid yellow area, and a few small granules had appeared in the cytoplasm (Fig. 1A). Cytoplasmic fluorescence increased until at 20 hr both nucleus and cytoplasm contained very fine yellow particles

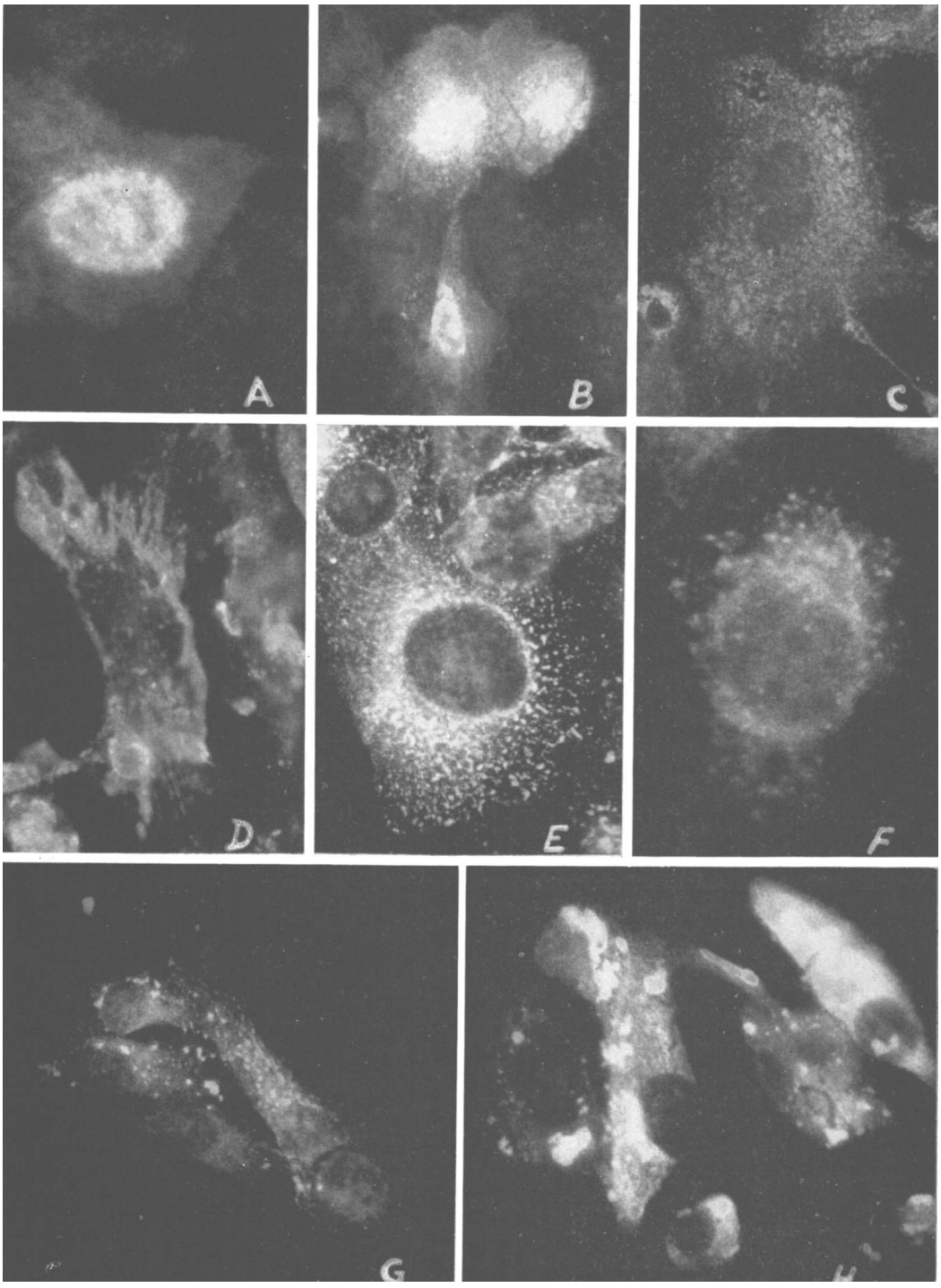

FIG. 1. A. Influenza virus in chick embryo lung cell. Intranuclear antigen at 8 hr. ×1000. B. Influenza virus in chick embryo lung cells at 20 hr. ×750. C. Sendai virus in chick embryo lung cell at 8 hr. ×450. D. Sendai virus in chick embryo lung cell at 26 hr. ×450. E. Sendai virus in human conjunctiva cells at 12 hr. ×450. F. NDV in chick embryo lung cell at 6 hr.

which imparted a dusty appearance to the cells and made it difficult in many cells to differentiate nucleus and cytoplasm (Fig. 1B). At 26 hr the nucleus had become relatively free of fluorescent material, but the cytoplasm contained many fine granules and a few larger fluorescent masses. Infectivity and hemagglutination titrations carried out on fluids from these cultures suggested that this represented a complete multiplication cycle. Measurable hemagglutinin levels were not reached in the 26 hr observation period but infectious virus release was first detectable at 12 hr and titers in the fluid had increased 200-fold by 26 hr.

When chicken embryo lung cells were inoculated with one EID_{50}/cell only about 10% of the cells produced antigen in the first cycle, but there was no essential difference in evolution of the pattern of antigen distribution from that seen with larger inocula.

Sendai virus. Chicken embryo lung cells were inoculated with 1000 EID_{50}/cell of Sendai virus previously maintained in the allantoic sac of the chicken embryo and cell preparations were collected for observation at 1, 2, 4, 6, 8, 12, 20 and 26 hr. Specific viral antigen was first visible in the cells at 2 hr as fine granules distributed throughout the cytoplasm. At 4, 6, and 8 hr (Fig. 1C) the cytoplasmic antigen steadily increased in quantity. At 12 hr some of the cells showed definite cytopathic effect. By 26 hr most cells contained large masses of cytoplasmic antigen (Fig. 1D) and many were rounded and contracted except for several long, fine cytoplasmic processes extending from each cell. The nucleus could be seen as a non-fluorescent area throughout the multiplication cycle. Hemagglutination and infectivity titrations performed on the fluids from these cultures suggested that the cycle of egg-adapted Sendai virus in chick embryo lung cells was largely incomplete. Very small amounts of infectious virus appeared in the culture fluid at 12 hr, but there was no further increase until 26 hr when cell degeneration was prominent, and even then titers of infectious virus reached no higher than $10^{-2.75}$. Hemagglutinin titer was 1:8 at 20 hr and 1:16 at 26 hr.

Study of a multiplication cycle of Sendai virus yielding complete infectious virus was made employing a virus line adapted to serial culture in human conjunctiva cells. Experiments showed that approximately 100% of cells in a culture of conjunctiva cells could be infected with an inoculum of 7 EID_{50} per cell. In one experiment the virus was diluted so that about 10% of cells were initially infected. After a latent period of 6 hr infected cells were first distinguishable by the presence of 15-20 small brightly fluorescent granules dispersed throughout the cytoplasm. The nucleus appeared as a clear, non-fluorescent area. At 8 hr the number and size of cytoplasmic particles of antigen had approximately doubled over that observed at 6 hr, but there was no change in general distribution of antigen. At 12 hr the cytoplasm was heavily stippled with antigen but the nucleus remained clear (Fig. 1E). By 24 hr the number of infected cells had increased to about 25%, apparently due to a second cycle of multiplication.

When conjunctiva cells were inoculated with 1000 EID_{50} per cell of egg-adapted Sendai virus all cells contained visible antigen at 2 hr after inoculation. At that time cells contained 20-40 small fluorescent particles scattered diffusely throughout the cytoplasm and no antigen was visible in nuclei. The number of cytoplasmic granules steadily increased and by 9 hr some larger masses of antigen had formed. Cytopathic effect was evident at 22 hr in formation of long cytoplasmic processes. At this time the entire cytoplasm was filled with large masses and fine granules of antigen. Throughout the multiplication cycle the nucleus could be distinguished by its lack of specific fluorescence.

In study of Sendai virus in conjunctiva cells it often was noted that cells in various stages of mitosis contained cytoplasmic antigen. Wheelock and Tamm(9) have similarly reported that antigen of influenza A and NDV

×900. G. NDV in chick embryo lung cells at 22 hr. ×450. H. Mumps virus in human conjunctiva cells at 26 hr. ×500. Direct observation suggested strongly that the small amounts of fluorescent material in nuclear area in C-H were due to overlying or underlying cytoplasmic antigen.

can be demonstrated in infected HeLa cells undergoing mitosis, although in fowl plague infection of chicken macrophages Franklin found cells in mitosis to be notably free of antigen(4).

Newcastle disease virus. Chick embryo lung cell cultures inoculated with 100 EID_{50}/cell were studied at 1, 2, 4, 6, 8, 12, and 22 hr after inoculation. Viral antigen was first visible in cells 4 hr after inoculation. Infected cells contained 20-30 small, brightly fluorescent granules distributed diffusely throughout the cytoplasm. By 6 hr an approximately 3-fold increase in number of granules of antigen had occurred, almost completely filling the cytoplasm with fluorescent material (Fig. 1F). At 8 hr the fluorescent material had begun to form larger aggregates which in some cases appeared to be located near the nuclear membrane but in other cells bore no distinct relationship to the nucleus. Observations at 12 and 22 hr showed no change in this pattern of fluorescence (Fig. 1G).

Chick embryo lung cells inoculated with 1 EID_{50}/cell developed no visible antigen until 8 hr after inoculation. The infected cells at this time contained 15-20 small granules which by 22 hr had increased in number to the point of filling the cytoplasm, resembling the pattern found at 6 hr in cultures inoculated with 100 EID_{50}/cell.

Mumps virus. Mumps virus was studied in 2 cell types. Chicken embryo lung cells were inoculated with the Enders strain (6 EID_{50}/cell) of egg-adapted virus. Since it was not certain that a complete multiplication cycle occurred in these cells, other experiments were performed using human conjunctiva cells and the Dunai strain of mumps virus adapted to serial culture in conjunctiva cells. Conjunctiva cells were inoculated with about one TCD_{50} per cell. With both cell types cultures were harvested and studied at 10, 15, 18, 22, 26, 38, and 65 hr after inoculation. Evolution of the cycle was essentially the same in both cell systems. At 18 hr after inoculation infected cells could first be recognized by the presence of 10-12 small yellow granules scattered throughout the cytoplasm. The nucleus could easily be discerned as a non-fluorescing area. By 26 hr antigen had increased to 5 or 6 large masses that occupied most of the cytoplasm (Fig. 1H). There were no further observable changes in quantity or distribution of antigen and at no time was fluorescence observed within the nucleus.

Discussion. It is clear that in sharp contrast to influenza virus the 3 non-influenza myxoviruses did not at any time in their multiplication cycle produce intranuclear antigen detectable with fluorescein-labeled antibody. The use of antisera from 3 species, employment of several multiplicities of infection, and use of 2 cell types indicate that the observed patterns of antigen development and distribution were not peculiar to a special set of materials or conditions. Ruling out such a possibility was important since in studies now in progress it has been found that under certain circumstances in at least one cell line an incomplete cycle of influenza virus multiplication is not accompanied by intranuclear antigen production although there is abundant cytoplasmic antigen. Additional supporting evidence pointing to lack of intranuclear antigen detectable by fluorescent antibody in cells infected with mumps virus or NDV can be found in several reports. Coons *et al.*(10) located mumps virus in the parotid gland of monkeys using mumps-convalescent monkey serum. Watson studied mumps infection of chicken embryos(11) and of chicken embryo tissues in culture(12) using convalescent monkey serum. Burnstein and Bang(13) identified NDV in infections of the nasal tract of chickens employing NDV-convalescent chicken serum. Wheelock and Tamm(9) made observations on NDV in HeLa cells and Prince and Ginsberg(14) studied the effect of NDV on Ehrlich ascites tumor cells using immune sera produced in rabbits. Most of these studies did not closely follow a single cycle of infection, and adequate levels of antibody against S antigen in their antisera were not confirmed by specific test, but most used antisera that would be expected to contain anti-S antibody and various parts of the multiplication cycle should have been present in their preparations. In all instances, however, anti-

gen was confined to the cytoplasm. In addition, Rountree(15) followed a single cycle of NDV infection in cultured chicken embryo cells using NDV-convalescent chicken serum, and throughout the cycle found antigen only in the cytoplasm.

While the present study indicates that intranuclear production of antigen demonstrable by fluorescent antibody is not characteristic of NDV, mumps, and Sendai viruses, it does not tell us where the S antigen associated with these viruses is produced. It remains possible that the nucleoprotein antigen is produced within the nucleus but in a form that does not combine with antibody under the conditions of the fluorescent antibody technic. On the other hand, it may be that in infections with these viruses S antigen is produced in the cell cytoplasm. Studies are in progress to clarify this point.

The finding that intranuclear antigen production so prominent in the multiplication cycle of influenza and fowl plague viruses is not demonstrable in the cycle of NDV, Sendai, and mumps viruses may add another characteristic to those that seem to separate the myxoviruses into 2 distinct subgroups. The site of antigen production in the cell has not been studied for all the myxoviruses, however, so that evaluation of these observations in this regard must await further information.

Summary. The multiplication cycles of Sendai, mumps, and Newcastle disease viruses were compared with that of influenza A virus in regard to site of antigen production in the cell. Single-cycle infections were followed using fluorescent antibodies prepared from infection-convalescent immune sera shown to contain anti-S and anti-V antibodies. In cells infected with influenza A virus antigen was first detectable in the nucleus and became abundant there by the time antigen appeared in the cytoplasm. In cells infected with Sendai, mumps, or Newcastle disease viruses earliest detectable antigen was found as small granules scattered throughout the cytoplasm. Antigen granules increased in size and number to form large cytoplasmic aggregates and masses. Multiplication of Sendai, mumps, or Newcastle disease viruses did not include production of antigen in the nucleus detectable by fluorescent antibody at any stage of the cycle.

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