jections of 200 γ thyroxin.‡ Injections were continued for 28 days or until death, whichever occurred sooner. All animals fed the synthetic ration (diet A_2) and receiving the thyroxin injections survived the experimental period of 28 days. All animals fed activated pancreas (diet C_2) and administered thyroxin died (average survival time 11.6 days). These findings indicate that the deleterious effects of pancreas feeding in the thyroid-fed rat were not due to increased digestion or absorption of thyroactive material.

‡ Thyroxin (Synthetic Cryst.), Roche-Organon, Inc., Nutley, N. J. The material was dissolved in .1 N NaOH, adjusted to a pH of 8.0 and diluted to a volume containing 200 γ thyroxin per cc.

Summary. Immature female rats failed to survive when fed purified rations containing both pancreas and desiccated thyroid. Length of survival was significantly prolonged if either pancreas or thyroid were eliminated from the experimental ration. The deleterious effects of pancreas in the thyroid-fed rat were not due to increased digestion or absorption of thyroactive material.

16638

Production of Hemagglutinin by Mumps and Influenza A Viruses in Suspended Cell Tissue Cultures.*

THOMAS H. WELLER AND JOHN F. ENDERS.

From the Research Division of Infectious Diseases of the Children's Hospital, Children's Medical Center, Boston, and the Departments of Pediatrics, and Bacteriology and Immunology, Harvard Medical School.

The property of agglutinating red blood cells characteristic of certain viruses such as mumps and influenza has provided a simple method of estimating their concentration in infected materials. The procedure however, insofar as we are aware, has not been applied to viruses propagated in tissue cultures. Accordingly, experiments were undertaken with the purpose of ascertaining (a) whether the mumps virus would proliferate in suspended cell tissue cultures of the Maitland type and (b) whether this virus and influenza A virus would in such media produce detectable levels of hemagglutinin.

Methods. Preparation and maintenance of cultures. Cultures were prepared in a manner similar to that described by the Maitlands.[1] The nutrient medium consisted routinely of 3 parts of a balanced salt solution made up according to the formula of Hanks† and 1 part of Simm's ox-blood serum ultrafiltrate.‡ This mixture will hereafter be referred to as "HS." In the experiments with mumps virus, the tissue employed consisted of amniotic membrane from 8- or 9-day-old chick embryos. The membranes were suspended in a small amount of "HS", minced with scissors to obtain fragments approximately 1 mm in diameter, and centrifuged for 2 minutes at 800 r.p.m. in a graduated centrifuge tube. The fragments were then resuspended in a total volume of "HS" six times that of the sedimented tissue. Using a large bore Pasteur pipette, 3 drops of this

* Aided in part by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Maitland, H. B., and Maitland, M. C., *Lancet*, 1928, **2**, 596.

† The balanced salt solution as recommended by Dr. J. H. Hanks, contained the following ingredients expressed as g/l: NaCl, 8.0; KCl, 0.4; $MgSO_4 \cdot 7\ H_2O$, 0.1; $MgCl_2 \cdot 6\ H_2O$, 0.1; $CaCl_2$, 0.14; Na_2HPO_4, 0.06; KH_2PO_4, 0.06; glucose, 1.0. To each liter, 5 cc 0.4% phenol red was added. After autoclaving at 10 lb. for 10 minutes, 0.5 cc 1.4% isotonic $NaHCO_3$ was added per 20 cc of the above.

‡ Purchased from Microbiological Associates Laboratories, Flemington, N. J.

tissue suspension were transferred to 25 cc Erlenmeyer flasks containing 3 cc of "HS" and fitted with rubber stoppers. In the experiments with influenza virus, the cultures were similarly prepared employing amniotic membrane, allantoic membrane, or embryonic brain tissue obtained from 8-day-old eggs. All cultures were incubated at 35°C.

Their subsequent handling differed from that usually employed with suspended cell cultures in that the nutrient fluid was replaced at intervals as indicated below. This was accomplished by tilting the flasks, allowing the tissue to settle, and removing as completely as possible the supernatant fluid from each flask with a fine bore Pasteur pipette; 3 cc of fresh "HS" were then added. In certain of the experiments subcultures were carried out by transferring aliquots of the pooled supernatant fluids thus removed to flasks containing freshly prepared tissue fragments.

Determination of hemagglutinin and egg infectivity titers. Using a modified Salk technic[2] the hemagglutinin titers were determined of the stock viruses and of the individual supernatant fluids removed periodically from the tissue cultures. The tests on the supernatant fluids were performed as soon as they were removed, using two-fold dilutions and beginning with a final dilution of 1:2. Pooled erythrocytes obtained from the same 4 hens were employed throughout. The highest final dilution of the supernatant fluid giving complete agglutination was taken as the endpoint. The results were expressed as a geometric mean of the individual hemagglutinin titers of the supernatant fluids from each set of cultures.

In the experiments on the growth of mumps virus in tissue cultures, the infectivity for chick embryos of the original inoculum and certain of the supernatant fluids removed at various times was determined. Aliquots of the supernatant fluids were pooled and centrifuged for 15 minutes at 1000 r.p.m. and the sediment discarded. Serial ten-fold dilutions

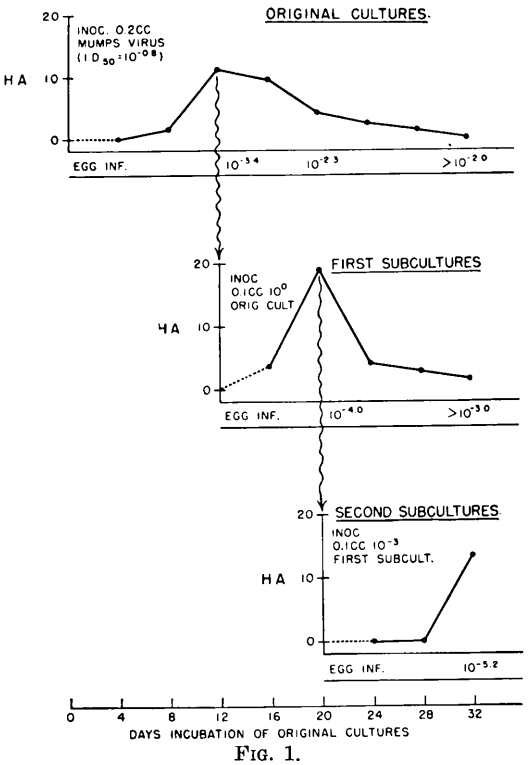

FIG. 1.

Mean hemagglutinin and infectivity titers of tissue cultures inoculated with mumps virus.

The hemagglutinin titer (HA) is expressed as the reciprocal of the geometric mean of the individual titers of the supernatant fluids from 4 cultures. The infectivity titers are expressed as the dilution of the pooled supernatant fluids from 4 flasks representing the ID_{50} dose for embryonated hen's eggs calculated according to the method of Reed and Muench.[3]

were then prepared in isotonic phosphate buffer (pH 7.1) and 7-day-old eggs in groups of 5 or 6 were inoculated intraamniotically with 0.1 cc of each dilution. Six or 7 days later, the amniotic fluids were harvested and tested individually in a final dilution of 1:8 for the presence of hemagglutinins. The presence of complete hemagglutination in this dilution was taken as evidence of infection. From the data so obtained the 50% infectivity end point was calculated by the method of Reed and Muench.[3] No assay of the infectivity of the cultures inoculated with influenza virus was made.

Experimental. (1). *Cultivation of mumps*

[2] Enders, J. F., and Levens, J. H., In: Diagnostic Procedures for Virus and Rickettsial Diseases. American Public Health Association, New York, 1948, pp. 139-164.

[3] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

virus. A typical experiment which is summarized in Fig. 1 will be described. The mumps virus employed as the initial inoculum consisted of pooled amniotic fluid derived from the 42nd egg passage of a strain originally isolated and maintained in this laboratory (Enders strain). This material had a hemagglutinin titer of 512 and an ID_{50} of $10^{-3.8}$ in eggs. Six tissue cultures were prepared with minced amniotic membrane. Four flasks were each immediately inoculated with 0.2 cc of a 10^{-3} dilution in isotonic phosphate buffer of the virus. The two uninoculated cultures were kept as controls. At 4-day intervals the supernatant fluids were removed and 3 cc of fresh "HS" were then added. In this way the original cultures were maintained over a 32-day period. The hemagglutinin titers of all the individual supernatant fluids were determined and the egg infectivity titers of the pooled supernatant fluids removed on the 12th, 20th and 32nd day after inoculation were established.

During this period of 32 days two subcultures were carried out. After the original cultures had been incubated 12 days, with two changes of "HS" in the interim, equal volumes of the supernatant fluids removed on the 12th day were pooled, centrifuged for 15 minutes at 1000 r.p.m. and the sediment discarded; 0.1 cc of the undiluted pooled supernatant fluid was then added to each of 4 newly prepared flasks containing minced amniotic membrane and "HS". The supernatant fluids from the control flasks were handled in an identical manner and 2 new control cultures set up. Flasks of the first subculture group were maintained 20 days with changes of the supernatant fluids at 4-day intervals.

The second subcultures were inoculated with material removed from the first subcultures on the eighth day of incubation. The supernatant fluids from the latter were pooled and centrifuged, diluted 10^{-3} in isotonic phosphate buffer and added in a volume of 0.1 cc to each flask containing newly prepared tissue. The second subculture flasks were incubated for 12 days, with change of the supernatant fluids every 4 days.

(2) *Cultivation of Influenza A virus*. Cultures prepared with minced amniotic membrane, chorioallantoic membrane or brain were inoculated with 0.1 cc of infected allantoic fluid containing the PR8 strain of Influenza A virus diluted 10^{-2} in isotonic phosphate buffer. In one experiment, flasks of each type of tissue were inoculated immediately after they were prepared, while to others the virus was added only after an interval of 6 days. In another experiment the virus was introduced 4 days after the cultures had been prepared and the supernatant fluid changed once. At various intervals, usually each day, 0.5 cc of the supernatant fluid was removed and replaced with the same volume of fresh "HS." The concentration of hemagglutinin in each specimen of fluid was determined.

Results. 1. Mumps virus. The results of the hemagglutinin and infectivity titrations carried out on the supernatant fluids removed from the tissue cultures are summarized in Fig. 1.

From these data it is evident that the mumps virus rapidly increases in cultures of this type. The original cultures were inoculated with 0.2 cc of diluted stock virus having a calculated ID_{50} titer for egg embryos of $10^{-0.8}$; thirty-two days later the ID_{50} titer of the pooled supernatant from the second subculture was $10^{-5.2}$. Not taking into account the quantity of virus discarded in the supernatants and assuming that each routine addition of nutrient fluid resulted in a 1:15 dilution of the virus, it may be calculated from the data included in Fig. 1 that an increase of the order of 1.3×10^{17} occurred in the egg infectivity titer. Likewise, during the experimental period an increase in the hemagglutinin titer of the order of 3.3×10^{14} occurred.

Active tissue growth of apparently the same degree was observed in all flasks irrespective of whether or not the virus had been inoculated. About the 6th to 8th day of cultivation free-floating, thin-walled cyst-like structures became numerous in the cultures of amniotic membrane. The cyst-like forms were no longer apparent after the 20th day. Between the 12th and 16th day, fixation of some of the tissue fragments to the bottom of

the flask was noted. These could be seen under low magnification to be surrounded by a zone of proliferating cells. In the cultures maintained 32 days there was an apparent decrease in the rate of growth after the 28th day; at this time many of the fragments previously adherent to the glass became loose and floated in the fluid.

Morphologic evidence of growth was accompanied by a fall in pH. Thus, during the 4-day interval between the changes of nutrient fluid, the pH, as estimated colorimetrically, fell from 7.6-7.7 to 6.9-7.1. This shift occurred throughout each 4-day period until the 28th day of cultivation in the original series. Thereafter, coincident with the apparent decrease in the rate of growth, the pH did not go below 7.3-7.5.

2. *Influenza virus.* The results with 3 different series of cultures have shown that the PR8 strain of Influenza A virus multiplies rapidly in the type of tissue culture employed as indicated by the early appearance and subsequent increase of hemagglutinin in the supernatant fluid. Thus in one experiment in which freshly prepared cultures of allantoic membrane, amniotic membrane and brain were employed, the geometric mean hemagglutinin titers after 72 hours of 2 cultures of each kind of tissue were 91, 6, and 32 respectively. Since the hemagglutinin titer of the virus inoculum was 512 and the dilution factor 3000, it can be calculated that the concentration of hemagglutinin during the period of cultivation had increased 535 times in the cultures of allantoic membrane, and 188 X and 35 X respectively in those of brain and amniotic membrane. The comparatively low titer obtained with the cultures of amniotic membrane should not be regarded as evidence for the failure of the virus to multiply as actively in this tissue, since in other experiments the amount of hemagglutinin produced was equivalent to that encountered in cultures of allantoic membrane and brain. In this instance the fact that relatively small amounts of amniotic tissue were employed probably was responsible for the low yield of hemagglutinin. It is of interest to observe that the embryonic brain tissue supported the multiplication of the virus almost as well as the allantoic membrane. Whereas neurotropic strains of influenza virus have been developed by Stuart-Harris and others[4–7] it is generally held that the PR8 strain has no affinity for nervous tissue.

Similar results were obtained in respect to the increase in hemagglutinin in tissue cultures which had been maintained for 4 or 6 days before the virus was inoculated. On the whole, however, the titers were somewhat lower. For example, 72 hours after inoculation of the virus, the geometric mean titers of two cultures of each of the 3 kinds of tissue were 8, 11, and 6 for allantoic membrane, amniotic membrane and brain respectively. These cultures had been prepared 6 days before the virus was added. Similarly in cultures of allantoic membrane prepared 4 days before inoculation the mean hemagglutinin titer did not exceed 16 after 72 hours.

Determinations of hemagglutinin titers at 12, 16, 20, 24, 48 and 72 hours and at certain intervals thereafter showed that the maximum production was attained between the 2nd and 3rd day. The hemagglutinin was present, however, in low concentration as early as 16 hours. By removing on the 4th day following inoculation all the supernatant fluid and replenishing with fresh material, it was found that thereafter a slow increase in hemagglutinin may gradually occur. But the titer attained did not exceed 2 even after an interval of 10 days and this was observed only in a series of cultures that were inoculated with the virus immediately after their preparation.

Discussion. It has been demonstrated that an egg-adapted strain of mumps virus multiplies actively in tissue cultures consisting of fragments of amniotic membrane of the chick embryo suspended in a mixture of ox serum ultrafiltrate and balanced salt solution. In

4 Stuart-Harris, C. H., *Lancet*, 1939, **1**, 497.

5 Francis, T., Jr., and Moore, A. E., *J. Exp. Med.*, 1940, **72**, 717.

6 Henle, G., and Henle, W., *Science*, 1944, **100**, 410.

7 Vilches, A., and Hirst, G. K., *J. Immun.*, 1947, **57**, 125.

this medium its multiplication is sufficient to produce measurable quantities of hemagglutinin in the supernatant fluid even when small inocula are employed. It is therefore possible within certain limits to estimate with ease the capacity of the virus to multiply under these conditions. It has also been determined that Influenza A virus (PR8 strain) increases in this type of medium to a level at which its hemagglutinating property can also be readily assayed.

The development of measurable amounts of hemagglutinin in tissue cultures is of significance, since it provides a swift and convenient means of following the rate and degree of multiplication of the mumps and influenza viruses in a system which, compared with a medium such as the chick embryo or the mouse, is far less complex. The technic, therefore, might be applied profitably to the study of many problems, for example, the effect of various chemical compounds in accelerating or inhibiting the growth of these viruses.

It is noteworthy that the rate of multiplication of influenza A virus was found to be much more rapid than that of mumps. This difference has been previously observed in experiments with infected chick embryos and would seem, as Ginsberg, Goebel and Horsfall[8] have indicated, to reflect a basic difference in the biologic properties of the two agents.

Conclusions. 1. An egg-adapted strain of mumps virus has been cultivated in suspended cell tissue cultures. 2. In such media the mumps virus as well as Influenza A virus (PR8 strain) produce measurable quantities of hemagglutinin.

[8] Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., *J. Exp. Med.*, 1948, **87**, 385.

16639

Magnesium Deficiency in the Sexually Mature Rat.*

G. P. BARRON, P. B. PEARSON, AND S. O. BROWN.

From the Department of Biochemistry and Nutrition and the Department of Biology, Agricultural and Mechanical College of Texas, College Station.

That the young rat requires an adequate dietary intake of magnesium for normal life processes was first established by Kruse, Orent, and McCollum.[1] As shown by Tufts and Greenberg, two phases seem to be observed in magnesium deficiency in young rats.[2] The first is marked by vasodilatation, hyperemia, and hyperexcitability. The second phase is characterized by nutritive failure, cachexia, and kidney damage. There have been controversial reports as to the pathological involvement of the skin in magnesium deficiency in rats. Kruse and his co-workers reported definite trophic changes in the skin and nails of young animals on a diet deficient in magnesium. Likewise, other workers reported skin lesions in animals deprived of magnesium.[3,4] On the contrary, Tufts and Greenberg and others did not observe disturbances in the skin of young animals raised on diets deficient in magnesium.[5,6] Greenberg suggested that the trophic conditions observed by several workers were due to the experimental animals not receiving various constituents of the vitamin B complex in sufficient quantities.[7]

* Aided by a grant from the Dow Chemical Company, Freeport, Texas, through the Texas A & M Research Foundation.

[1] Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1932, **96**, 519.

[2] Tufts, E. V., and Greenberg, D. M., *J. Biol. Chem.*, 1938, **122**, 693.

[3] Watchorn, E., and McCance, R. A., *Biochem. J.*, 1937, **31**, 1379.

[4] Shrader, G. A., Prickett, C. O., and Salmon, W. D., *J. Nutrition*, 1937, **14**, 85.

[5] Brookfield, R. W., *Brit. Med. J.*, 1934, **1**, 848.