*turus viridescens*. The present study also reveals that retention over a period of 7 days, when measured by the savings technique, was equal for diploid and tetraploid salamanders, a somewhat surprising result in view of the tetraploid's poor learning ability.

At this point, it appears safe to conclude that polyploidy, whether diploid or tetraploid, brings about a decrease in maze learning ability. It is not possible, however, to state whether such an effect is the result of the increase in cell size, the reduction in number of cells, or the reduction in number of neural connections that probably results from the reduced number of cells.

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26 February 1957

## Observations on Local Spread of Pox Viruses in Tissue

In the course of experimental work on the local spread of pox viruses in the infected chorioallantoic membrane of the embryo, we frequently encountered (*1*, *2*) peculiar ring zones consisting of concentric rings around a central focus of infection (Fig. 1). In plants, ring-zone formation has been known for a long time. The ringspot viruses, which cause concentric necrotic and non-necrotic areas on infected leaves, are typical examples. As far as we know, similar findings with animal viruses have not been reported. The ring zones appear to be a suitable model for the study of the mode of local spread of virus in infected tissues. This report is a brief summary of our observations on this problem.

Our experiments were carried out with two strains of dermovaccinia, one of neurovaccinia, one of cow pox, one of variola, and two of fowl pox virus. All strains had been passaged in eggs more than ten times. Embryonated 10-day eggs were inoculated on the chorioallantoic membrane according to a previously described modification of Burnet's method (*1*, *3*). The amount of seed virus varied between 10 and 1000 minimal infective doses, and the membranes were harvested at various intervals before and after the death of the embryos. Our experimental material comprised nearly 1000 eggs. For macroscopic examination, the membranes were placed in Kaiserling solution and imbedded in glycerine gelatine. For histological study, they were fixed in phosphate-buffered formalin (4.37 percent by weight of formaldehydus solutus, *p*H 7.4) and imbedded in paraffin. The sections were stained with hematoxylin and eosin.

Macroscopically typical ring zones were found around both the primary and the secondary foci, the latter appearing after the generalization of the infection (Fig. 1).

The number and the intensity of the zones depended on the time of survival of the host: the longer the embryo survived, the more marked the ring zones. The zones were always more distinct and more constant with fowl pox than with the other pox viruses examined. This may be owing to the different character of the fowl-pox infection. The chorioallantoic picture, the much longer incubation period, the lower virus content of the affected tissues, the relatively high survival rate of the infected embryos, and the slower course of the infection differentiate fowl pox from the more active vaccinia, cow pox, and variola viruses in incubated eggs. For this reason, histological examinations were carried out only with fowl pox-infected chorioallantoic membrane.

The results obtained show that the ring zones in fowl pox-infected chorioallantoic membrane are caused by stepwise centrifugal spreading of the virus in the tissue. The phenomenon is the result of a complex process in which both the proliferating ectoderm with its concentric exfoliations and the entoderm with its concentric rampartlike proliferations play a part. The latter are particularly prominent at the periphery of the foci (Fig. 2).

There can be little doubt that the ectodermal exfoliations are primarily caused by the virus, while the viral cause of the entodermal proliferations is proved by the characteristic vacuolizing degeneration of the cells (Fig. 2). There is no fundamental difference between the primary and the secondary foci.

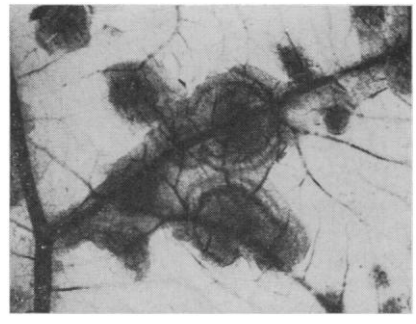

Fig. 1. Ring-zone formation around an older fowl-pox focus in the chorioallantoic membrane. Two dim rings show around the pocks. (×6)

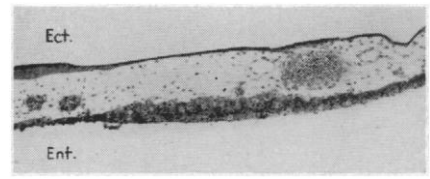


Fig. 2. Peripheral ring zone of a secondary focus of fowl pox. The rampartlike entodermal proliferation (Ent.) extends far beyond the altered zone of the ectoderm (Ect.). (×73)

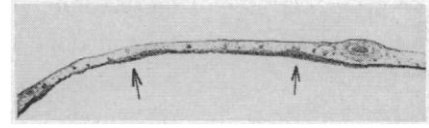

Fig. 3. Cross-section through two rings at the periphery of a fowl-pox focus. Note that such rings are caused by entodermal proliferation exclusively (arrows point to the thickened entodermal areas). (×27½)

The appearance of rampartlike, almost undulated entodermal proliferations around some foci strengthens the concept of a local centrifugal spread of the virus from cell to cell connected with rhythmical differences in viral activity. In the course of spreading, the pathogenicity of the virus appears to be exhausted and restored periodically. Zones of high virus activity and marked tissue damage alternate with zones of low viral activity and little or no pathogenic effect.

This hypothesis of rhythmic virus spread is strengthened by the presence of shaggy ectodermal proliferations that appear at almost regular intervals. The extent of these proliferations is greatly reduced toward the periphery of a focus.

Of special interest in this connection is the fact that, in the course of generalization, the secondary virus implantation in the chorioallantoic membrane begins in the entoderm. In the ectoderm, there is extensive viral spread in the early stages of focus formation (Fig. 3). Later, especially in secondary foci, the peripheral proliferating ramparts in the entoderm extend far beyond the last ectodermal alteration (Fig. 2).

A striking feature of this intermittent local spread of virus is the fact that the intensity decreases toward the periphery and finally stops. In other words, the local spread of virus does not proceed unlimitedly, but is checked at a certain point. The reason for this phenomenon

is not known. To find the correct explanation would be an important contribution to a better understanding of the virus-cell relationship.

The possibility that the ring zones are the result of mesodermal cellular infiltration or that they are caused by external nonspecific stimulations or by antigen-antibody reactions could be excluded. On the other hand, it is not unlikely that the formation of ring zones is influenced to some extent by the alternating physiological condition of the eggs. This hypothesis is supported by the observation that pox viruses settle better in viable embryos than they do in fertilized eggs of low vitality. In the latter, the secondary foci do not show the same degree of development as in more viable eggs after the same period of incubation.

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11 February 1957

## Hemagglutination Test for Toxoplasmosis

The *in vitro* dye test of Sabin and Feldman (*1*) is at present the most useful diagnostic procedure for toxoplasmosis. However, it has difficulties in its performance which make it unsuitable for use in most routine diagnostic laboratories. It requires live parasites, which are dangerous to handle, and it requires microscopic reading of the tests, large quantities of an accessory factor that is found in normal human serums and that is sometimes difficult to obtain, and considerable care in most of the manipulations involved. The reports of Middlebrook and Dubos (*2*), Boyden (*3*), Keogh *et al.* (*4*), Alexander *et al.* (*5*), Stavitsky (*6*), and others have demonstrated the applicability of the hemagglutination procedure to a variety of antigens, both polysaccharide and protein in nature. The technique has already been tested in parasitic infections, such as schistosomiasis and trichinosis, by Kagan (*7*). In an effort to devise a practicable test for the diagnosis of toxoplasmosis, we have explored the applicability of this procedure.

A number of *Toxoplasma* antigens have been prepared and tested in the hemagglutination test, using the following procedure. Toxoplasmas are harvested from the peritoneal exudates of intraperitoneally infected mice into a tared tube containing saline, buffered at *p*H 7.2. The harvested organisms are centrifuged and the supernatant discarded. A volume of sterile distilled water 10 times the wet weight of the sediment is added, the sediment is resuspended, and the mixture is allowed to stand at 5°C for at least 18 hours, with occasional shaking. The solid particles are then removed by centrifugation, and an equal volume of 1.7-percent saline is added. The antigen now constitutes approximately a 1/20 (weight/volume) dilution of the original parasite harvest but contains only the water-soluble components of the parasites. It is stored in the deepfreeze until it is used.

Sensitization of red cells with these antigens requires treatment of the red cells with 1/20,000 tannic acid. Sheep red cells have been used, and the techniques are like those used by Stavitsky (*6*). The red-cell concentration used was close to that recommended by Stavitsky. The density of the red-cell suspension was measured in each case by lysing 1 ml of the diluted cells with 5 ml of distilled water and measuring the optical density at 520 mμ in a Beckman spectrophotometer. It was found that the most usable concentrations gave readings varying from 0.4 to 0.6. A diffuse settling of cells is regarded as a 4+ reaction, whether or not any crinkling of the outer edges of the settled film occurs. The 2+ reaction of Stavitsky is the same as our 2+ reaction, and is taken as the endpoint, at which the titer of the test serum is measured.

Block tests have been performed to determine more closely the optimal concentrations of reagents to use in the hemagglutination test. In general, the results indicate that an antigen concentration of 1/200 and a red-cell suspension giving an optical density reading of 0.4, as described, gave the highest titers with the test serums, with adequate negative controls. A reading of 0.4 is obtained with a suspension of 2.0 to 2.5 percent red cells, depending on length of storage. It appears also that antigens vary in their initial potency, and each one must be adjusted for optimal concentration prior to use.

Table 1. Agreement between dye-test titers and hemagglutination titers in tests with different lots of erythrocyte-sensitizing antigens.

| Dye-test titer | No. of tests | Hemagglutination tests on same serums agreeing within | |
|---|---|---|---|
| | | 2-fold difference | 4-fold difference |
| 1/16 | 11 | 7 | 11 |
| 1/64 | 12 | 9 | 12 |
| 1/256 | 9 | 7 | 9 |
| 1/1024 | 10* | 4 | 6 |

* All tests agreed within an eightfold difference.

About 15 *Toxoplasma* antigens have been tested by this procedure, using a variety of human serums, either pooled or individual specimens, with different dye-test titers. Not all antigens have been used with the same serums. Good agreement has been found between dye-test titers and hemagglutinating titers, as seen in Table 1. Fourfold differences in titer can frequently be found in different dye tests on the same serums. Therefore, the hemagglutination test agrees as well with the dye test as can be expected. The hemagglutination reaction, in tests on serums from one proved human case of lymphadenopathic toxoplasmosis, became positive slightly later than the dye test, but much earlier than the complement-fixation test.

Results thus far obtained in more than 100 tests similar to those described in Table 1, but using pooled lots of antigen, continue to show excellent agreement between the hemagglutination and dye tests. All except one serum found positive with the dye test have been positive also by hemagglutination. Agreement between the tests within a four-fold difference in titer has been obtained with 93 percent of the specimens. With only 4 percent of the serums was the disagreement in titer greater than 16-fold. All of this work has been done with human serums. While additional problems may be encountered with animal serums, it seems justifiable to conclude that the hemagglutination reaction offers promise of providing a practicable serological procedure for the diagnosis of toxoplasmosis.

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