1. Weber, G., Allard, C., DiLamirande, G., Cantero, A., *Endocrinol.*, 1956, v58, 40.
2. Ashmore, J., Hastings, A. B., Nesbett, F. B., Renold, A. E., *J. Biol. Chem.*, 1956, v218, 1956.
3. Nimni, M. E., Carminatti, H., Dellacha, J. M., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 690.
4. Good, C. A., Kramer, H., Somogyi, M., *J. Biol. Chem.*, 1933, v100, 485.
5. Sorensen, N. S., *Nature,* 1957, v180, 857.
6. Shull, K. H., Ashmore, J., Mayer, J., *Arch. Biochem. and Biophys.*, 1956, v62, 210.
7. Rosen, F., Roberts, N. R., Nichol, C. A., *J. Biol. Chem.*, 1959, v234, 476.
8. Gavasto, F., Pileri, H., Brusca, A., *Biochim. et Biophys. Acta,* 1957, v24, 250.
9. Long, C. N. H., Katzin, B., Fry, E., *Endocrinol.*, 1951, v26, 309.
10. Hess, W. C., Shaffran, I. P., *J. Wash. Acad. Sci.*, 1957, v47, 17.
11. Marks, P. A., Horecker, B. L., *J. Biol. Chem.*, 1956, v218, 327.
12. Leloir, L. F., Cardini, E. E., *J. Am. Chem. Soc.*, 1957, v79, 6340.

Received January 20, 1960. P.S.E.B.M., 1960, v103.

## Zone Electrophoresis Studies on Hemagglutinin of Hemagglutinating and of Masked Strain of Polyoma Virus. (25639)

ROBERT CRAMER AND SARAH E. STEWART (Introduced by J. W. Beard)
*Institut de Recherches sur le Cancer G. Roussy, Villejuif, Seine, France, and Nat. Cancer Inst., Bethesda, Md.*

Eddy *et al.*(1) demonstrated an hemagglutinin in mouse embryo tissue culture fluids infected with polyoma virus. This hemagglutinin was lacking in cultures with no tumor-producing capacity. It was not possible to dissociate hemagglutination from the cytopathogenicity and tumor-inducing capacity by various methods such as heat treatment, ether treatment, limiting dilution titration, and first passage plaques from limiting dilutions. The authors, therefore, considered hemagglutination to be an inherent characteristic of the tumor-inducing agent. Kahler *et al.*(2) found in electron microscopic studies of purified polyoma virus an apparently uniform population of particles with a diameter of 40-45 mμ. By sedimentation experiments in a zonal density gradient particle counts were found to parallel the hemagglutination titer. However, no data on the relation of number of particles observed to possible tumor-inducing capacity are given. Hartley and Rowe (3) showed that culture fluids with high tumor-inducing capacity might fail to produce hemagglutination. It could be demonstrated, however, that unmasking of the hemagglutinin could be achieved either by heat or RDE treatment. It seemed desirable to study the interrelation of the different biological attributes of mouse polyoma issue culture fluids by methods other than sedimentation. Preliminary results of electrophoretic studies on the hemagglutinin both of hemagglutinating and of non-hemagglutinating (masked) Polyoma strains are given. Comparable studies on electrophoretic mobility of tumor-inducing capacity, and of cytopathogenicity are in progress.

*Materials and methods. Virus.* Strain 1000 with a hemagglutination titer of 1/128 and strain 8844 with a titer ¼ both from the Nat. Cancer Inst. were used. Both strains were known to give about 100% early tumor formation in Swiss mice. Virus was grown in 2% horse serum in medium 199. For unmasking the hemagglutinin of strain 8844, heat treatment for 30 to 60 min. in 56°C water bath was used. *Electrophoresis technic.* A detailed description of the electrophoresis apparatus and technic has been given(4,5,6). Tissue culture fluids were treated with one cycle of centrifugation for 10 min at 6000 g in a Spinco rotor 40. The supernate was dialysed against the buffer used for electrophoresis and mixed with the appropriate quantity of cane sugar to fit the density conditions for introduction into the electrophoresis column. Veronal buffer of pH 8.6 containing 205 g sodium diethylbarbiturate and 28 g diethylbarbituric acid in 10 l of distilled water was

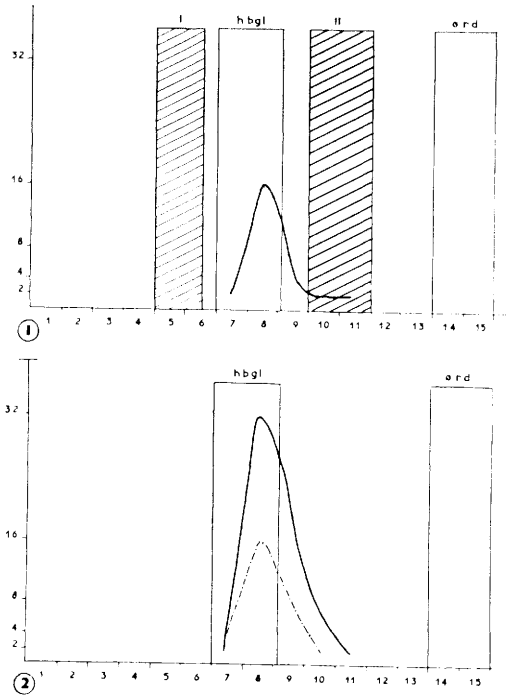


FIG. 1. Zone electrophoresis diagram showing distribution of Polyoma hemagglutinin activity of strain 1000 (continuous line). Ha titer indicated on ordinate, consecutive electrophoretic fractions of 0.5 ml each on the abscissa. Reference substances rabbit hemoglobin (hbgl) and neutral red (ørd). Impurities: opalescent (I) and turbid zone (II).

FIG. 2. Electrophoresis diagram indicating mobility of hemagglutinin of strain 1000 (continuous line) and of unmasked strain 8844 (broken line) in relation to mobility of rabbit hemoglobin.

used. Electrophoresis was allowed to proceed for 2 to 3 hours at room temperature with a voltage gradient of 3.5 V/cm and a current of 15 m A. Experiments with and without addition of reference substances were made. Rabbit hemoglobin and neutral red served as reference substances. After completion of electrophoresis, the contents of the column were run out by a separate sampling capillary and divided into fractions of 0.8 ml or less. The hemagglutination titer of each fraction was tested by the pattern method(7), using 0.3 ml from each fraction added to 0.3 ml of a suspension of 0.25% guinea pig erythrocytes.

*Results.* Invariably 2 visible zones of "impurities" appear in all experiments (Fig. 1). A first opalescent zone (zone I, Fig. 1) migrates definitely slower than rabbit hemoglobin and separates from it. A second very sharply defined zone of turbidity (zone II, Fig. 1) appeared above the hemoglobin zone, equally well separated. A minor turbid zone not indicated in Fig. 1 migrated slightly more slowly than neutral red.

No major hemagglutination activity was found corresponding to either of these zones described. The major peak of hemagglutination appeared invariably between zone I and zone II, a zone occupied by rabbit hemoglobin in the experiments in which this substance had been added as reference substance.

As the hemagglutinin assumed the same position relative to zone I and zone II in experiments without addition of rabbit hemoglobin, the mobility recorded seems to be characteristic for polyoma hemagglutinin.

About 50 to 70% of hemagglutinating activity was recovered in each electrophoresis experiment.

It is evident that major impurities appearing in crude preparations can be separated by zone electrophoresis from the hemagglutinin; these impurities must correspond to undefined substances, probably released at the moment of cell lysis in the culture fluid. A part might be due to the 2% horse serum added to the original culture medium.

It was intended to compare mobility of the hemagglutinin of strain 1000 to mobility of the hemagglutinin of the masked strain 8844.

According to Hartley and Rowe, a certain extent of unmasking could be achieved by heat treatment. In our experiments, exposure at 37° for 30 min was ineffective whereas with exposure at 56° for 30 to 60 min a certain extent of unmasking could be achieved with strain 8844. A remasking occurs when the sample is stored at 4°, which confirms the findings of Hartley and Rowe.

The hemagglutinin of the unmasked 8844 strain assumed the same position in the electrophoresis column as hemagglutinin of strain 1000 (Fig. 2). As electrophoresis was not extended over more than 3 hours, possible minor differences in electrophoretic mobility could have escaped observation. The relatively short time of electrophoresis was compensated by increasing inspection density *i.e.*, in sampling as small volumes as are compat-

ible to test the hemagglutination. This seemed justified in sugar gradient zone electrophoresis because of the great stability against convection offered in this system.

Some electrophoresis experiments were done to test the possibility that a definite virus-inhibitor dissociation could be achieved in zone electrophoresis. For unmasking strain 8844, treatments with potassium periodate or RDE that would destroy or inactivate the inhibitor were avoided. The reason for this is that only preparations in which the inhibitor is preserved can be used for testing the possibility that a definite hemagglutinin-inhibitor dissociation may be achieved by zone electrophoresis. For this purpose, in some experiments, zone I (Fig. 1) of opacity was carefully removed by the first capillary that served to introduce the sample into the electrophoresis column. The hemoglobin zone containing the hemagglutinin was then removed separately by a second capillary, care being taken to finish sampling at a moment when the lower boundary of zone II (Fig. 1) just appeared above the tip of the second capillary. The hemagglutination titer of the purified hemagglutinin fraction was tested at once. At the same time, the hemagglutinin titer of the control sample of unmasked 8844 strain that had been used in electrophoresis but had been kept at the temperature of the room where the experiment was performed, was likewise tested for hemagglutination. To test the remasking effect, an aliquot of unmasked, not electrophoresed and unmasked "purified virus" was kept for 24 or 48 hours at 4°. After this time, both were again tested for hemagglutination using the same guinea pig erythrocytes in the same concentration.

Provided the stability of the hemagglutinin in the purified state is the same as observed in crude preparations, a comparison of hemagglutination titers of control and electrophoretically purified hemagglutinin before and after remasking treatment should indicate whether or not an inhibitor-hemagglutinin dissociation can be achieved by zonal electrophoresis.

One such experiment gave the results indicated in Table I.

TABLE I. Unmasking of 8844 Strain by Heat Treatment and Effect of Electrophoresis on Remasking Effect.

| | HA titer | | | |
|---|---|---|---|---|
| 8844 | Before unmasking | After unmasking | Before remasking at completion of electrophoresis | After remasking |
| Control | 1/8 | 1/64 | 1/64 | 1/8 |
| Electrophoresed | 1/8 | 1/64 | 1/32 | 1/32 |

The masked strain 8844 was used. For this strain, obtained from the Nat. Cancer Inst., a HA titer of ¼ was found. When the sample was retested one month later after having stayed at –70°, a titer of ⅛ was recorded. It is not clear if during this time some inhibitor action was lost. After heating at 56° for 60 min., an unmasking was found, the HA titer being 1/64. A remasking of the control sample was observed after treatment at 4° for 24 hours. No remasking was observed with electrophoresis fraction 8, as the same HA titer of 1/32 was observed whether the fraction was tested immediately after completion of electrophoresis or after having been for 24 hours at 4°. The possibility has been considered that the absence of remasking of purified electrophoresed virus might be due to the saccharose environment of the electrophoresis column rather than to a definite virus-inhibitor dissociation. Therefore, virus was added to saccharose in veronal buffer to give a final dilution of 20% and an aliquot was added to the same amount of veronal buffer. An initial titer of 2 was found. After heating for 1 hour at 56°, unmasking to a titer of 64 occurred. In both the saccharose and the veronal samples remasking to a titer of 2 occurred after staying at 4° for 24 hours. Another cycle of unmasking and remasking with the 2 samples gave the same result. The saccharose environment, therefore, can not be responsible for the unmasking. A definite dissociation between the hemagglutinin and its inhibitor by electrophoresis seems to be the most plausible explanation.

*Summary.* 1) Polyoma virus hemagglutinin of a hemagglutinating strain 1000 and of a masked strain 8844 were examined by zonal electrophoresis in a density gradient of cane sugar. 2) The masked strain 8844 was un-

masked by heat treatment previous to electrophoresis. 3) Both hemagglutinins migrated with the same speed slightly faster than rabbit hemoglobin when tested after an electrophoresis for 3 hours at pH 8.6. 4) Support for the presence of an inhibitor of hemagglutinin in the masked strain 8844 was gained. 5) It appeared that a hemagglutinin-inhibitor separation could be achieved when previously unmasked hemagglutinin was purified by electrophoresis.

1. Eddy, B. E., Rowe, W. P., Hartley, J. W., Stewart, S. E., Huebner, R. J., *Virology,* 1958, v6, 290.
2. Kahler, H., Rowe, W. P., Lloyd, B. J., Hartley, J. W., *J. Nat. Cancer Inst.,* 1959, v22, 647.
3. Hartley, J. W., Rowe, W. P., *Virology,* 1959, v7, 249.
4. Cramer, R., Lerner, K. D., Polson, A., *Sci. Tools,* 1957, v4, 1.
5. Polson, A., Cramer, R., *Biochim. Biophys. Acta,* 1958, v29, 187.
6. Cramer, R., *Nature,* 1959, v183, 195.
7. Eddy, B. E., Rowe, W. P., Hartley, J. W., Stewart, S. E., Huebner, R. J., *Virology,* 1958, v6, 290.

Received January 25, 1960. P.S.E.B.M., 1960, v103.

## Demonstration of Immune Response to Foot-and-Mouth Disease Vaccine In Protection Test in Young Adult Mice. (25640)

RAYMUNDO G. CUNHA (Introduced by M. E. Wegman)
*Pan American Foot-and-Mouth Disease Center,* Rio de Janeiro, Brazil*

Since its development in 1938, aluminum hydroxide adsorbed foot-and-mouth disease virus vaccine(1,2) has gained wide acceptance throughout the world. Potency tests of this vaccine are generally conducted in cattle, the number used varying according to the quality of the test(3,4,5). The high cost of the test and the difficulty in infected countries of obtaining susceptible cattle generally make impracticable the potency testing of each batch of vaccine. It would be highly desirable, therefore, to find a laboratory animal that could be used as a substitute for cattle in such tests. Recent publications show that this problem is being investigated elsewhere. Uhlmann and Traub(6) refer to use of suckling mice to determine immune response of vaccinated adult mice and Skinner(7) refers to use of adult mice, the immunity of which is tested by antibody titration in suckling mice (as cited by Henderson,5). The experience that foot-and-mouth disease virus could be adapted to adult mice(8), led to investigation of the immune response of these animals to aluminum hydroxide adsorbed vaccines, assessing the immunity established by response to inoculation of the vaccinated mice with adapted strains of virus. Brief reference to this work has already been made(9) and in the present paper a description is given of experiments performed to evaluate the protection produced in young adult mice by foot-and-mouth disease vaccines prepared from virus of different sources and to determine if the protection afforded was type specific.

*Materials and methods.* A Rockefeller Institute strain of Swiss mice was used. Those referred to as suckling mice were 6 to 8 days old. The young adult mice were weaned mice, 3 to 6 weeks old, weighing 9 to 13 g at beginning of experiments. Older mice, up to 8 weeks and 21 g weight, were also used for certain preliminary experiments with type O vaccines. Aluminum hydroxide formalised vaccines were used that had been prepared in the State Veterinary Research Inst., Amsterdam, Holland; the Veterinary Research Inst., Maracay, Venezuela; the Animal Biol. Inst., Rio de Janeiro, Brazil or in Pan American Foot-and-Mouth Disease Center. The virus for vaccine preparation was obtained by the Frenkel method of culture(10), by inoculation of the tongue of cattle or by infection of

*Project of Pan American Sanitary Bureau financed by Technical Cooperation Program of Organization of American States.