mouse tumor, determined by any one method, only the average and range of results for each type of sample have been listed in Table I. The data of Mickelson and Barvick(5) has been included for purposes of comparison. The values for rat tumor obtained in this laboratory by microbiological assay and column chromatography are in excellent agreement except for glutamic acid, glycine, and proline which are slightly higher by the chromatographic method. The values determined by paper chromatography differ considerably from those by the other 2 methods but in many cases are of the same order of magnitude.

The small difference in aspartic acid content observed in the microbiological assay data for the 2 types of tissue is confirmed by column chromatography, while that for 6 other amino acids is not confirmed (Table I). The lack of agreement with the results of Mickelson and Barvick(5) may stem from a difference in the method of hydrolysis employed. Although the absolute values differ with the method used, it may be concluded from the results of both paper and column chromatography that there is no significant difference in total amino acid composition between the rat and mouse tumors.

Summary. 1. Percentages of total amino acids in viable tissues of the Walker carcinosarcoma 256 (rat), Sarcoma 180 (mouse), and Taylor tumor (mouse) have been determined by both column and paper chromatography. 2. Values obtained by column chromatography agreed satisfactorily with those determined in the same laboratory by microbiological assay while the majority of values determined by paper chromatography were in poor agreement with those of the other two methods. 3. There was no significant difference in the total amino acid content of tumors from rats or mice.

1. Baginsky, M. L., Murphy, E. A., Dunn, M. S., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 427.

2. Baginsky, M. L., Thesis, Univ. of Calif., at Los Angeles, 1958.

3. Dunn, M. S., Feaver, E. R., Murphy, E. A., *Cancer Res.*, 1949, v9, 306.

4. Sauberlich, H. E., Baumann, C. A., *ibid.*, 1951, v11, 67.

5. Mickelson, M. N., Barvick, L., *J. Nat. Canc. Inst.*, 1956, v17, 65.

6. Greenstein, J. P., *Biochemistry of Cancer*, Academic Press, Inc., New York, 1954.

7. Roberts, E., Tanaka, T., *Cancer Res.*, 1956, v16, 204.

8. Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A., Reiner, P. J., *Univ. of Calif. Publ. in Physiol.*, 1949, v8, 293.

9. Moore, S., Stein, W. H., *J. Biol. Chem.*, 1951, v192, 663.

10. Ishii, S., *J. Biochem.* (Japan), 1956, v43, 531.

11. Chinard, F. P., *J. Biol. Chem.*, 1952, v199, 91.

12. McFarren, E. F., Mills, J. A., *Anal. Chem.*, 1952, v24, 650.

13. Baker, B. E., Khan, N. A., *J. Sci. Food Agri.*, 1957, v8, 217.

Received April 6, 1962. P.S.E.B.M., 1962, v110.

Studies on Measles Viral Hemagglutination.* (27555)

J. R. PERIES† AND C. CHANY‡ (Introduced by R. J. Huebner)
(With technical assistance of Mrs. Catherine Baquey)
Centre de Recherches sur les Virus, Hopital Saint Vincent de Paul and Service des Virus, Institut Pasteur, Paris

The authors have demonstrated(1) the hemagglutinating (Fig. 1) and hemolytic activities of measles virus. Hemagglutination was specifically inhibited by convalescent sera but not by sera from persons with no previous his-

* This study was supported by grant No. E-2235 of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, U.S.A.

† Research Fellow in receipt from the University of Buenos Aires. Present address: Istituto Nacional de Microbiol., Buenos Aires, Argentina.

‡ Charge de Recherches, Inst. Nat. d'Hygiene, Paris.

tory of measles infection. The hemagglutination-inhibition (HI) test was applied subsequently to diagnostic and epidemiological problems concerned with measles in several laboratories(1,2,3,4,5). The existence of a small non-infectious particle was later reported(6). Such particles appear with infectious virus in infected cell cultures. Both the non-infectious and infectious viral particles are antigenic and agglutinate monkey erythrocytes.

The composition of the measles tissue culture antigen and the mechanism of red cell agglutination are reported here.

Materials. The Edmonston strain of measles virus, which had been adapted to growth in KB tissue culture by Dekking and McCarthy(7), was used for preparation of the antigen. The KB cells were grown in 500 ml Roux bottles with growth medium that consisted of 100 ml of casein hydrolysate with 5% horse serum and a maintenance medium which contained only 2% horse serum. In some experiments, Roux bottles of 3.5 liter capacity were used with 300 ml of medium. The monolayers were infected with virus at a multiplicity of 50. After incubation at 36.1°C for 7 to 10 days, typical lesions were observed in approximately 50% of the cells. The cells were scraped from the glass and the whole culture fluid was centrifuged at 3000 rpm for 5 minutes. The sedimented cells were ground in a Potter grinder and resuspended in the supernatant fluid. The whole culture fluid was recentrifuged to remove cell debris, and the supernatant fluid was concentrated 10-fold by dialysis with polyvinylpyrrolidone powder at 4°C, as follows. A dialysis "bag" which contained the supernatant fluid was placed in a vessel. Polyvinylpyrrolidone powder (Rhône-Poulenc) was spread over the outside of the membrane to extract the water. A weighted vessel was placed on top of the membrane to keep the fluid under continuous pressure. In some instances, concentration was effected by pervaporation with a technic described by Colin and Kaplan (personal communication). In this procedure, the dialysis membranes were attached to the frame of an oscillating fan which had been tilted upwards. Continuous moistening was accomplished in this way. After concentration by either method, the preparation was redialyzed against a phosphate buffered saline solution of pH 7.0. In recent experiments(8), monkey kidney cells which had been grown at 40.5°C in casein hydrolysate medium with 10% horse serum were used. The cells were maintained in casein hydrolysate medium without serum. Monkey kidney cells which had been grown at 40.5°C produced measles agglutinating antigen at a workable titer without any concentrating procedure.

Methods. Cynocephalus monkey red cells were collected in Alsever's solution, or more often, in sodium citrate solution. Cells were washed 3 times with saline and a final cell concentration of 0.5% in saline was prepared. In some experiments, erythrocytes from *Erythrocebus patas* monkeys were used with satisfactory results, and Rosen(3) has used rhesus monkey erythrocytes.

For assay of hemagglutinin (HA), 0.2 ml of 0.5% red cells were added to 0.2 ml of serial 2-fold dilutions of the antigen. The tests were performed in plastic plates with dilution of the antigen in saline. When cells had sedimented after incubation at 37°C, the titration endpoint was read as the highest dilution in which complete (4+) hemagglutination was observed.

Hemagglutination inhibition. Since some inactivated (heated at 56°C for 30 minutes) rabbit and human sera contain agglutinins active against monkey erythrocytes, the sera used in these tests were absorbed with an equal volume of 50% monkey erythrocytes by incubation at 4°C overnight. Sera were centrifuged to remove erythrocytes, then tested for presence of erythrocyte agglutinins. The HI test was performed by addition of 0.1 ml of antigen, diluted to contain 4 HA units per ml, to 0.1 ml of serial 2-fold dilutions of serum (starting at 1:2) in plastic plates. One-tenth ml of 1% cynocephalus red cell suspension was added, and the test was incubated at 37°C for approximately 30 minutes. The endpoint was considered to be the serum dilution which completely inhibited hemagglutination.

Infectivity titrations were performed utilizing KB cell suspensions (60,000 cells per

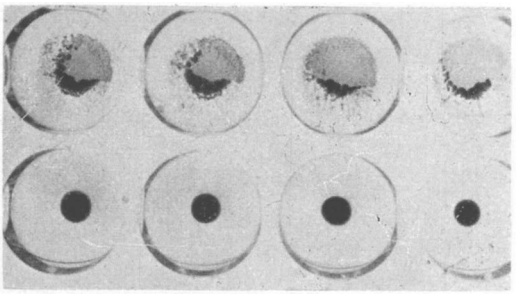

FIG. 1. Upper line shows hemagglutinating pattern of measles viral antigen with baboon red cells. Lower line shows control with uninoculated but concentrated tissue culture material.

ml). The 50% endpoint was calculated after 10 days using the method of Kärber.

Results. The hemagglutinating activity of measles-infected tissue culture fluid has been demonstrated(1) with only monkey erythrocytes (Fig. 1). Table I gives the results of comparative tests of the adsorptive capacity of cynocephalus monkey and guinea pig erythrocytes for measles virus. After incubation for one hour at 37°C nearly all of the hemagglutinating virus had been adsorbed to the simian red cells while HA titer of the fluid adsorbed with guinea pig red cells remained unchanged. Parallel infectivity titrations indicated that almost all of the infectious virus was removed from the culture fluid by monkey but not by guinea pig erythrocytes.

Some of the more relevant biophysical properties of the hemagglutinin were also studied (Table II). The HA was incubated with crystalline pancreatic trypsin at final concentrations of 62.5, 125, 250 and 500 γ per ml for 1 hour at pH 8.0. The data of Table II show that the HA was destroyed by the lowest concentration of the enzyme used. We reported earlier(1) that proteolytic enzymes failed to destroy hemagglutinating activity, but these results apparenlty can be attributed to the low potency of the enzyme preparations then available. Similarly, the HA was incubated with crystalline RNA-ase and with DNA-ase at final concentrations of 100 μg per ml. No significant reduction of HA titer was observed. Tests of the specific activities of the enzymes were performed for us by Drs. J. Hupper and J. Pelmont(9). The effect of ether was tested with the method described by Delsal(10) as follows: Equal proportions of 5% ethyl alcohol in reagent-grade ether and the hemagglutinin were mixed and agitated for 10 minutes at 4°C. The mixture was then frozen in a dry ice-alcohol bath and quickly thawed. The ether was evaporated and nitrogen gas was allowed to bubble through the mixture to effect final removal of ether. This treatment significantly reduced the HA titer and completely destroyed the virus infectivity.

TABLE I. Hemagglutinin and Infectivity of Measles Infected Tissue Culture Fluid Before and After Adsorption with Monkey and Guina Pig Erythrocytes.

Erythrocyte used for adsorption	Hemagglutinin titer		Infectivity titer ($TCID_{50}$ per ml)	
	Before	After	Before	After
Monkey	64	0	$10^{4.25}$	$10^{0.25}$
Guinea pig	64	64	$10^{4.25}$	$10^{3.75}$

TABLE II. Eect of Trypsin, RNA-ase, DNA-ase and Ether Treatment on Measles Virus Hemagglutinin.

Treatment of hemagglutinin or antigen	Hemagglutinating titer before treatment	Hemagglutinating titer after treatment
Trypsin (62 γ ml)	32	< 2
RNA-ase	128	128
DNA-ase	128	128
Ether	64	8

Incubation of the measles antigen for 2 hours at 37°C or its storage at 4°C for at least 3 months did not significantly reduce the HA titer. However, incubation at 60°C for 20 minutes showed that although infectivity of the preparation was not detected after 10 minutes, hemagglutinating activity was not significantly decreased. After an additional 40 minutes at this temperature, a 4-fold loss in titer was observed(Fig. 2).

Nature of hemagglutinin. Comparison of the hemagglutinin and infectivity titers showed that in many cases the amount of infectious virus detected by titration was too small to explain the agglutination of erythrocytes. Therefore, it was postulated that hemagglutination might be caused by some other factor in the virus preparations, in addition to the infectious virus. Further ex-

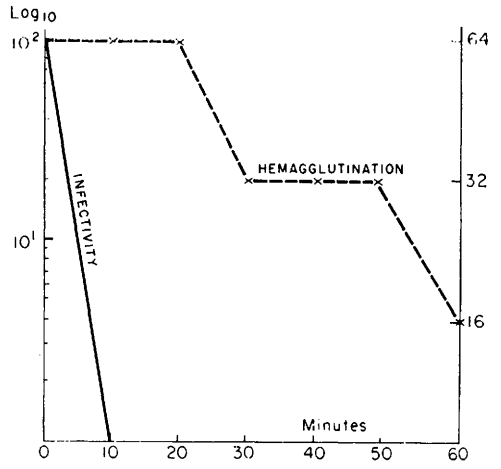

FIG. 2. Dissociation of infectivity and hemagglutinating activity of measles viral antigen by heating at 60°. The low infectious titer at beginning of this experiment contrasting with relatively high hemagglutinating titer is probably partly due to loss of infectious activity during concentration.

periments with ultrafiltration and ultracentrifugation demonstrated the presence of a small hemagglutinating but non-infectious particle. The presence of the infectious and non-infectious particles was demonstrable in measles-infected cell culture but not in non-infected control cultures. The data of Table III show that when the virus preparation was filtered through 25, 47 and 56 mμ gradacol or Millipore membranes, HA titer was practically unchanged, but that nearly all infectious virus was retained by the membranes. Adequate controls showed that the membranes were not altered during the filtration. In Exp. 5 (Table III), infectious culture fluid was filtered successively through 100 mμ Millipore and 47 mμ gradacol membranes. Some activity was retained by the former but all the unretained HA passed through the latter membrane.

The hemagglutinating and infectious titers of the 10-fold concentrated and dialyzed antigen preparation were studied before and after centrifugation in the No. 40 rotor of a Spinco model L preparative centrifuge. Four different speeds were used for a time interval of one or 2 hours. Supernatant fluid from only the upper fourth of each tube and the sediment were tested. The data of Table IV show that reduced but significant hemagglutinating activity persisted although practically all infectious virus was sedimented. It was possible to separate the "large" and "small" hemagglutinating units by centrifugation. In all experiments where this separation was effected, the ratio between small non-infectious units and large infectious units was approximately 1:8 to 1:16. Table V shows results of HI tests of the small and large particle hemagglutinins with hyperimmune measles serum. The two kinds of particles were inhibited by the serum at slightly different dilutions. It can be concluded that the smaller particles are equally antigenic and are more heat labile than the complete larger particles. Their ability to hemagglutinate was lost after 24 hours at 4°C.

Nature of erythrocyte attachment. It has been reported(1) that the virus does not elute spontaneously from the red cells when held

TABLE III. Filtration Experiments Through Graduated Membranes.

Exp. No.	Filter	Hemagglutinating titers Before	After	Infectious titer ($TCID_{50}$ per ml) Before	After	Remarks
1	Gradacol, 25 mμ	8	8	10^2	0	Antigen diluted 1/10
	" , 47 "	8	8	10^2	0	*Idem*
	" , 56 "	8	8	10^2	0	"
	Millipore, 10 "	64	0	—	0	Undiluted antigen
2	Gradacol, 56 mμ	16	8	$10^{3.75}$	0	*Idem*
3	Millipore, 50 mμ	128	32	—	—	"
4	*Idem*	64	64	$10^{1.12}$	$10^{0.75}$	Fluid collected following washing of upper side of filter
5	Millipore, 100 mμ	32	8	$10^{3.75}$	0	Successive
	Gradacol, 47 "	8	8	0	0	filtration

TABLE IV. Centrifugation of 10-Fold Concentrated and Dialyzed Measles Antigen at 4 Different Speeds.

Exp. No.	Time, hr	Speed, rpm	xg	Hemagglutinating titer: Before	Hemagglutinating titer: After sediment	Hemagglutinating titer: After upper ¼ of tube	Infectious titer: Before	Infectious titer: After sediment	Infectious titer: After upper ¼ of tube
1	1	15,000	20,360	64	32	16	10^3	$10^{4.75}$	$10^{2.50}$
2	1	20,000	26,360	128	128	16	$10^{2.75}$	10^4	$10^{1.25}$
3	2	30,000	59,310	16	16	0	$10^{2.99}$	$10^{2.37}$	$10^{1.37}$
4	2	40,000	100,000	64	128	undiluted	10^4	$10^{4.75}$	$10^{1.00}$

at room temperature for 48 hours. At 37°C, elution was not observed after 4 hours at which time the red cells began to hemolyze. If the agglutinated cells were resuspended by shaking the tubes, hemagglutination occurred after resettling of the cells and this process could be repeated. Red cells which had been agglutinated by the A2 (Asian) strain of influenza virus and from which the virus had eluted were still agglutinable by measles virus. However, they were refractory to agglutination with fresh influenza virus.

The hemolytic activity of all receptor-destroying enzyme preparations for monkey red cells prevented experiments to determine the nature of erythrocyte receptors. Formalin-treated cells, which have been used by others(11,12), were studied with measles virus. The data of Table VI show that formalin-treated red cells are not agglutinated by measles virus while the titer obtained with influenza virus with such cells is similar to the titer obtained with untreated cells.

Discussion. The experiments presented here show that measles viral particles can be adsorbed on to the surface of appropriate monkey red cells, while they are not adsorbed to guinea pig red cells; the latter are not agglutinated by measles virus. Measles hemagglutinating antigen is completely destroyed by a proteolytic enzyme such as trypsin. RNA-ase or DNA-ase on the other hand have no detectable effect. Ether reduced significantly, but did not completely destroy, hemagglutinating activity. Heating at 60°C destroyed infectivity in 10 minutes while hemagglutinating activity was more slowly reduced. The decrease in activity became significant (4-fold decrease) after one hour.

TABLE V. Inhibition of Hemagglutination of Large HA Units and Small HA Units with Specific Immune Sera.

	Large HA units	Small HA units
Exp. 1	1:768	1:386
2	1:1920	1:960

TABLE VI. Hemagglutination of Formalin-Treated Red Cells by Measles and Influenza A2 Viruses.

Virus	Hemagglutinating titer: Control	Hemagglutinating titer: Formalin-treated red cells
Measles	128	0
Influenza A2	256	512

In the light of these experiments, the lack of parallelism between the infectious titer and the hemagglutinating titer is best explained by the appearance in the cell cultures of a non-infectious but hemagglutinating factor, consisting at least in part of small particles which can, like mature virus, adsorb on to red cells. Since hemagglutination due to the small particles is also inhibited by human antiserum these particles are also antigenic, are more heat labile and do not induce any cytopathic effect. The small particles are produced during infection of the cell with measles virus. In the cell culture system used in the present studies (KB, monkey kidney) the ratio of non-infectious small hemagglutinating units to the infectious and hemagglutinating units was consistently around 1:8 to 1:16. It is possible that the tissue culture antigen may also contain large non-infectious hemagglutinating units. Apparently, the small hemagglutinating units can better be demonstrated when the method of concentration described here is applied. It is likely that this method will be useful for study of the hemagglutinating and antigenic properties of other animal viruses.

The biochemical characterization of the measles antigen with trypsin sensibility, ether sensitivity and resistance to RNA-ase and DNA-ase suggest that the antigen consists mainly of lipoproteins. These lipoproteins are probably constituents of the viral capsid.

It can be postulated that the small particles are debris of unused constituents appearing during viral synthesis.

The receptors for measles virus are destroyed by formalin treatment of red cells. Erythrocytes previously agglutinated by myxoviruses are still agglutinable by measles virus. These findings show that receptors for measles virus are different from the receptors for myxoviruses. In addition, measles virus does not elute spontaneously from the red cells unlike myxoviruses.

Summary. Hemagglutination of monkey red cells was demonstrated with a concentrated measles tissue culture antigen. The antigen was destroyed by trypsin and its activity was significantly reduced by ether. Heat inactivation at 60°C for 10 minutes destroyed the infectivity, while hemagglutinating activity was significantly reduced only after one hour. A small non-infectious hemagglutinating unit was also demonstrated. Both hemagglutinating particles are antigenic and their activity was inhibited by specific antiserum. The small particles appeared during viral multiplication in the cell. Receptors on the red cells were of a different nature than those of myxoviruses.

The authors are indebted to Drs. L. Rosen, J. Kern and R. Huebner for help and criticism in preparation of this manuscript.

1. Peries, J. R., Chany, C., *Compt. rend. des Scéances de l'Acad. des Sci.,* 1960, v251, 820.
2. Tournier, P., personal communication, 1960.
3. Rosen, L., *Virology,* 1961, v13, 139.
4. Rosanoff, E. I., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 563.
5. Demeio, J. L., Gower, T. A., *Virology,* 1961, v13, 367.
6. Peries, J. R., Chany, *Compt. rend. des Scéances de l'Acad. des Sci.,* 1961, v252, 2956.
7. Dekking, F., McCarthy, K., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 1.
8. Chany, C., Thomas, M., *Compt. rend. des Scéances de l'Acad. des Sci.,* 1961, v253, 579.
9. Hupper, J., Pelmont, J., personal communication, 1960.
10. Delsal, J. F., *Bull. Soc. Chim. Biol.,* 1949, v31, 122.
11. Flick, J. A., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 448.
12. Fauconnier, *Ann. Inst. Pasteur,* 1958, v95, 775.

Received April 6, 1962. P.S.E.B.M., 1962, v110.

Localization of Anti-Rat Kidney Antibodies in New-Born Rats.* (27556)

R. HIRAMOTO, P. CALCAGNO, AND D. PRESSMAN
Department of Biochemistry Research, Roswell Park Memorial Institute, New York State Dept. of Health, Children's Hospital, and University of Buffalo, Buffalo, N. Y.

Experimental nephrotoxic nephritis has been demonstrated by many investigators to occur in rats injected with anti-rat kidney antisera prepared in rabbits. Calcagno and coworkers(1,2) have reported that new-born rats do not develop the clinical disease even when injected with 4 times the dose (on a weight basis) of rabbit anti-rat kidney serum required to produce disease in adult rats. At 2 weeks of age clinical disease could be produced with twice the dose required for adults. Moreover, if the rats injected as newborn were reinjected at 3 to 14 weeks of age only about half of them showed clinical disease although previously uninjected 6-week-old animals all develop the disease at the dosage given.

Since the new-born rats could not develop clinical disease when injected with nephrotoxic serum which was capable of inducing experimental nephritis in the adult rat, studies were carried out to determine whether

* Supported in part by grants from Nat. Heart Inst.