gold *in vitro*. Opsonin is suspected to be a "natural antibody" in view of the phagocytic stimulatory activity of plasma from the germ-free animals.

1. Benacerraf, B., Biozzi, G., Halpern, B. N., Stiffel, C., In: Physiopathology of the Reticuloendothelial System, Halpern, B. N., ed., Blackwell, Oxford, 1957, 52.

2. Zilversmit, D. B., Boyd, G. A., Bruccer, M., J. Lab. Clin. Med., 1952, v40, 255.

3. Dobson, E. L., In: Physiopathology of the Reticuloendothelial System, Halpern, B. N., ed., Blackwell, Oxford, 1957, 80.

4. Wilkins, D. J., Myers, P. A., J. Reticuloendothelial Soc., 1964, v1, 344.

5. Jenkin, C. R., Rowley, D., J. Exp. Med., 1961, v114, 365.

6. Filkins, J. P., Smith, J. J., Proc. Soc. Exp. Biol. & Med., 1965, v119, 1181.

7. Normann, S. J., Benditt, E. P., J. Exp. Med., 1965, v122, 709.

8. Saba, T. M., Di Luzio, N. R., J. Reticuloendothelial Soc., 1965, v2, 437.

9. Saba, T. M., Filkins, J. P., Di Luzio, N. R., ibid., in press.

10. Wright, A. E., Douglas, S. R., Proc. Roy. Soc. (London), 1903, Ser. B72, 357.

11. Rowley, D., J. Exp. Med., 1960, v111, 137.

12. Howard, J. G., Wardlaw, A. C., Immunol., 1958, v1, 338.

13. Manwaring, W. H., Fritschen, W., J. Exp. Med., 1923, v112, 1121.

14. Fenn, W. O., J. Gen. Physiol., 1921, v3, 575.

15. Suter, E., Ramsier, H., Adv. Immunol., 1964, v4, 117.

16. Filkins, J. P., Di Luzio, N. R., Proc. Soc. Exp. Biol. & Med., 1966, v122, 177.

17. ———, ibid., 1966, v122, 548.

18. Boyden, S., Internat. Rev. Exp. Pathol., 1963, v2, 311.

19. Di Luzio, N. R., Riggi, S. J., J. Reticuloendothelial Soc., 1964, v1, 136.

20. Zilversmit, D. B., Salky, N. K., Trumbull, M. L., McCandless, E. L., J. Lab. Clin. Med., 1956, v48, 386.

21. Doll, J. P., Am. J. Physiol., 1962, v203, 291.

22. Thorbecke, G. J., Benacerraf, B., Ann. N. Y. Acad. Sci., 1959, v78, 247.

23. Solomon, J. B., Immunology, 1966, v11, 97.

24. Bauer, H., Horowitz, R. E., Watkins, K. C., Popper, H., J.A.M.A., 1964, v187, 715.

Received March 13, 1967. P.S.E.B.M., 1967, v125.

Replication of Rubella Virus in a Continuous Line of African Green Monkey Kidney Cells (Vero).* (32167)

HARVEY LIEBHABER, JOHN T. RIORDAN, AND DOROTHY M. HORSTMANN
(Introduced by F. L. Black)
Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Conn.

Rubella virus has been cultivated in a variety of continuous mammalian cell cultures which develop cytopathic changes (CPE) as a consequence of virus infection(1-8). Such virus host cell systems have been tested in many laboratories engaged in the study of rubella virus. Thus far no single tissue culture system has been applied with uniform ease and advantage from one laboratory to another. Among the factors which probably contribute to this variability are: 1) CPE may be subtle and slow in developing, thus making its recognition and differentiation from non-specific changes difficult for the observer having little or no experience with a particular system. 2) the appearance and development of CPE is dependent on the composition of the nutrient medium used; for example, the rabbit kidney line LLC-RK-1 cells will develop CPE with certain lots of Medium 199 and not with others(9). Exactly how the lots differ from one another is not known. Leerhøy(10) has clearly shown the variations in morphology of uninfected SIRC cell cultures as well as the variation in appearance of CPE with different nutrient media.

The overall usefulness of the host cell systems in which CPE develops is further

* This work was aided by a Grant from The National Foundation and in part by USPHS Grant AI 07443-02.

limited since in general they require specifically adapted virus(6,11,12) and with the exception of the baby hamster kidney line BHK-21(5) do not yield virus in high titer.

Primary isolation of rubella virus (RV) is still considered to be most effectively accomplished in primary cultures of African green monkey kidney cells (pGMK)(13). Virus is detected by a state of interference in these cells to the cytopathic effects of a superinfecting virus. The interference method has also been used to measure RV neutralizing antibody. Though the method is responsible for a substantial body of information concerning RV, it is time consuming and laborious. Moreover, the measurement of neutralizing antibody by interference is an indirect test which is subject to the influences of a number of variables which limit reproducibility and precision(14,15).

Clearly no one tissue culture host system has been applied to the study of rubella virus in which a) CPE is obvious, specific in appearance, rapid in onset and regularly reproducible; b) primary virus isolation can be made with a frequency greater than the pGMK-interference system and c) virus is consistently grown to titers exceeding $10^6 ID_{50}$/ml.

It was within this frame of reference that the present study was undertaken. This report describes the properties of a continuous line of African green monkey cells (Vero) with regard to a) the development of cytopathic changes and plaques in response to RV infection, b) the comparative sensitivity of Vero cultures for primary RV isolation and c) the titers of infectious virus and complement fixing antigen obtained from Vero cultures.

Materials and methods. Media and solutions. The media used throughout this study were Eagle's minimal essential medium (MEM)(16), Medium 199(17) and a lactalbumin hydrolysate yeast extract medium (LHY), containing 1.7 g lactalbumin hydrolysate, 0.57 g yeastolate (Difco), 0.84 g $NaHCO_3$ and 1 g of additional glucose per liter of Earle's balanced salt solution (10). For the growth of cell cultures medium was supplemented with 5% fetal calf serum or 10% agamma newborn calf serum. For maintenance of test cultures media were supplemented with 2% fetal or agamma calf serum. Virus was diluted in phosphate buffered saline (PBS) containing 1% agamma calf serum. All sera were heat inactivated.

Tissue cultures. The continuous line of African green monkey (*Cereopithecus aethiops*) kidney cells (Vero) studied in these experiments was kindly supplied by Dr. N. Weibenga, Laboratory of Tropical Virology, National Institutes of Health. Vero cells were originally isolated by Dr. Y. Yasumura at the Chiba University in Chiba, Japan. Confluent monolayers in Roux bottles were washed with Puck's saline A, dispersed with saline A containing 0.125% trypsin and 0.01% versene, and suspended in MEM growth medium. For the preparation of roller tube cultures the cell suspension was diluted to contain 1.5×10^5 cells/ml. Tubes were seeded with 1 ml of suspension and incubated at 37°C for 3 days at which time the medium was changed. Cultures were ready for use 3 to 4 days later. For preparation of monolayers for plaque assay, 5 ml of cell suspension containing 3×10^5 cells/ml were delivered into 60 mm plastic petri dishes and incubated at 37°C in a humidified 5% CO_2 atmosphere. Confluent monolayers were ready in 3 to 4 days.

Virus. Strain Mag rubella virus, isolated in this laboratory during a local 1962 epidemic, had been through 17 serial passages in primary green monkey kidney and 5 serial passages in RK-13 cells before being cultured in Vero cells. Mag strain after passage in Vero cells is referred to as Mag-V, and the subscript following the V indicates the number of passages in Vero cultures. Dr. P. Parkman of the National Institutes of Health provided a sample of attenuated RV which had been through 77 serial passages in primary green monkey kidney cultures (HPV-77). Samples of Mag and HPV-77 grown in Vero, BHK-21 and RK-13 cultures were also studied.

Specimens for virus isolation. Serum and throat swabs were obtained 3 times weekly from chimpanzees during the course of experimentally induced rubella infection. Throat

swabs were collected in Hanks' BSS containing .5% bovine albumin. They were stored at —20°C until tested.

Infectivity titrations and neutralization tests. Titration of infectivity was performed by inoculating 0.1 ml of serial 3-fold or 10-fold dilutions of the test suspension into each of 4 drained culture tubes. After adsorbing for 1 hour at room temperature each tube received 1 ml of LHY medium with 2% fetal calf serum. Appropriate control cultures were included in each titration. Cultures were observed for CPE daily for 14 days at which time the test was concluded. Titers were calculated by the method of Kärber(18). A parallel titration in pGMK by the echovirus 11 interference method was run at the same time.

In neutralization tests 0.5 ml volumes of serial 2-fold dilutions of a test serum mixed with an equal volume of virus suspension estimated to contain 100 $TCID_{50}$ of virus/0.1 ml were incubated for 60 minutes at 4°C, and then inoculated in 0.2 ml amounts into each of 4 roller tube cultures. Simultaneous titration of the virus employed was included in every neutralization test. The cultures were observed for CPE after 4 days and daily thereafter. The test was concluded 8 days after the appearance of CPE in the virus control cultures. The neutralization titer was taken as the reciprocal of the highest serum dilution which completely inhibited CPE. Parallel titrations of the same sera were performed by the echovirus 11 interference method in pGMK(14).

Plaque assay. Confluent Vero monolayers were drained free of growth medium, inoculated with 0.2 ml of serial 10-fold dilutions of virus, and incubated at 37°C in a humidified 5% CO_2 atmosphere for 60 minutes with frequent rocking. The monolayers were then overlaid with 7 ml of medium consisting of equal parts of 1.9% Difco noble agar containing 200 μg of DEAE dextran/ml and double strength MEM containing 10% calf serum. A second overlay identical with the first was added after 5 days; after an additional 5 days a third overlay, without DEAE dextran containing 1:20,000 neutral red was added.

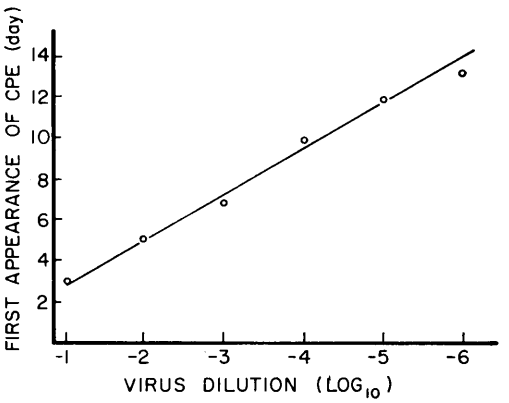

FIG. 1. Relationship between dilution of rubella virus inoculum and time of appearance of CPE.

Preparation of fixed and stained tissue culture specimens. Infected and control cultures on coverslips were washed 3× with PBS and fixed in Bouin's solution for 15 minutes. They were washed in 80% ethanol and subsequently stored in fresh 80% ethanol until stained with hematoxylin and eosin.

Results. Cytopathic effects (CPE). The time interval between infection and the appearance of CPE varied quite regularly with the concentration of virus inoculated. This relationship is illustrated in Fig. 1 in which the $\log_{10}$ of the dilution of virus inoculum is plotted against the time (in days following inoculation) at which CPE was first observed.

CPE began with the development of focal accumulations of piled up, dense, rounded cells. These foci degenerated and detached from the glass surface over a 2- to 3-day period, while new foci developed elsewhere in the monolayer. During periods of observation up to 21 days, complete degeneration of the cell sheet was not usually observed. Well advanced CPE was found to decrease significantly if infected cultures had more than one medium change during the period of observation.

Intense clumping of chromatin was the earliest change observed in fixed and stained preparations. This appeared to be followed by progressive condensation of chromatin and shrinking of the affected nuclei. The infected cell ultimately appeared round and shrunken with an intensely eosinophilic cytoplasm and a small dense basophilic nucleus. These

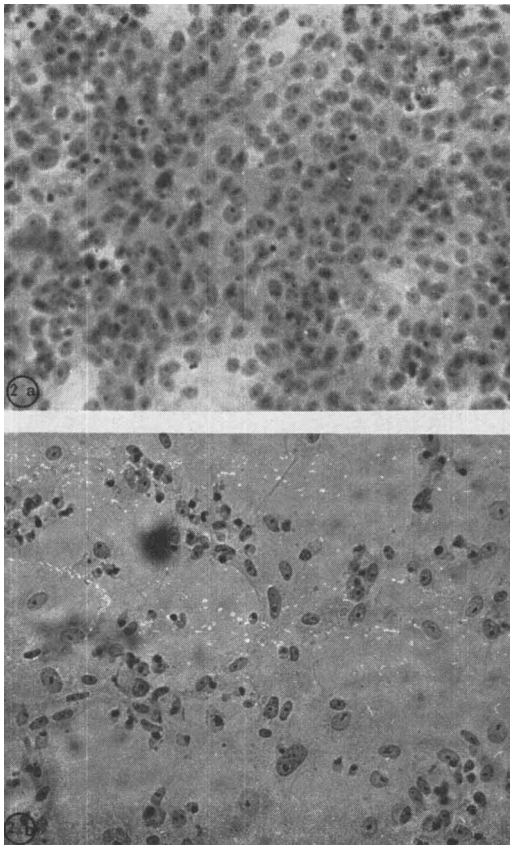

FIG. 2. Vero monolayers prepared at the same time from the same cell suspension. Fixed in Bouin's solution and stained with hematoxylin and eosin. Final magnification 400×. A) Uninfected control culture. B) Rubella virus CPE 4 days after infection.

changes are illustrated in Fig. 2. No inclusion bodies were observed.

That the changes which were interpreted as CPE were specific and related to the presence of virus rather than non-specific changes is supported by the following evidence. 1) A series of Vero tubes, some read as positive, some as negative at the end point of a titration, were individually tested for the presence of virus by passage into pGMK cultures which were challenged 9 days later with echovirus 11. The cultures inoculated with material from Vero tubes judged to show CPE resisted echovirus 11 challenge, whereas those inoculated with fluids from CPE-negative Vero tubes were completely destroyed by the echovirus 11 challenge. 2) Preimmune serum did not neutralize changes recognized as CPE whereas specific rubella immune serum did.

Growth of rubella virus in Vero monolayer cultures. Groups of 5 Vero monolayer cultures (136th passage) in 3 oz bottles were inoculated respectively with rubella virus (Mag-V_3) at input multiplicities of 0.001, 0.01 and 0.1 ID_{50}/cell. Five control bottles were inoculated with uninfected tissue culture fluid. All cultures were incubated at 37°C. Samples collected at various time intervals from each set of cultures were pooled and assayed for infectivity in pGMK cultures by the echovirus 11 interference method. The resulting growth curves are shown in Fig. 3. Peak virus titers between $10^{6.5}$ and $10^{6.8}$ ID_{50}/ml, appeared 6 to 8 days after infection. Virus titers in these cultures after 3 to 4 days seemed to be independent of the multiplicity of input virus over the range studied. In contrast, the sizeable differences in virus titers observed from the first through the third day of the growth cycle were in

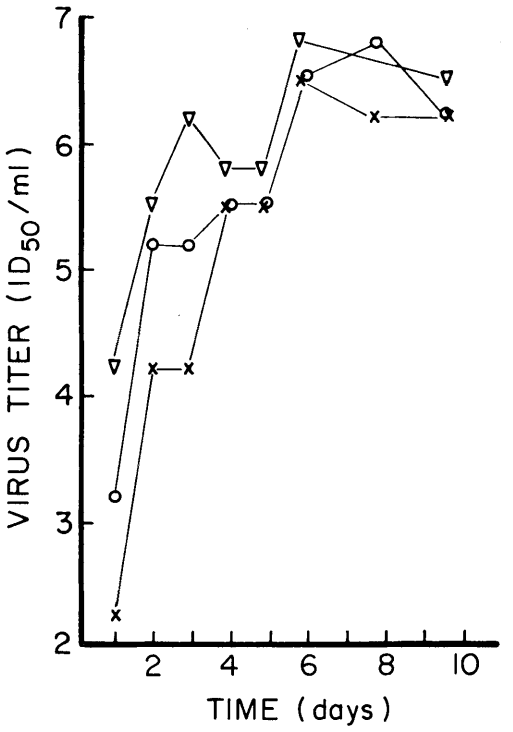

FIG. 3. Growth of rubella virus in Vero monolayers inoculated with various multiplicities of input virus. ▽——▽ 0.1 ID_{50}/cell; 0——0 0.01 ID_{50}/cell; ×——× 0.001 ID_{50}/cell.

TABLE I. Comparative Infectivity Titers of Rubella Virus in Vero and Primary Green Monkey Kidney Cultures.

Virus	Passage*	Infectivity titer (log_{10}/ml) Vero cells (by CPE)	pGMK cells (by interference)
Mag	$G_{11}R^7V_4$	6.75	6.25
"	$G_{11}R^7V_{12}$	7.0	6.5
"	$G_{11}R^7V_2B_2$	6.75	6.25
HPV	$G_{77}V_3$	6.75	6.75
"	$G_{77}R_1$	4.75	4.50
"	$G_{77}B_2$	7.0	6.50

* G = primary African green monkey kidney cells; R = RK-13 continuous rabbit kidney cells; V = Vero cells; B = BHK-21 cells. Subscript refers to No. of serial passages.

direct proportion to the differences in input virus multiplicity.

Sensitivity of assay in Vero cultures. A number of virus stocks which had been passed through various cell cultures were assayed in parallel infectivity titrations by CPE in Vero cultures, and by echovirus 11 interference in pGMK cells. The results are tabulated in Table I. Assuming that the factor responsible for CPE in Vero and interference in pGMK cultures are identical, it appears that the Vero cell is slightly but consistently more sensitive to the cytopathic potential of a given quantity of rubella virus than is the pGMK cell to the interference potential of the same quantity of virus.

Plaque formation. Both Mag-V_3 and HPV-77-V_2 produced plaques in Vero monolayers. Plaques generally appeared between 12 and 14 days and reached maximal numbers by the 18th day. They measured 4-5 mm in diameter and were round with indistinct borders. The plaques were not completely destained and consequently were difficult to read at times. A photograph of a plaque dish is presented in Fig. 4. Plaque counts increased linearly with increasing concentrations of the virus inoculated. Plaques were neutralized by convalescent human antiserum whereas acute serum did not cause plaque reduction. Plaque development was inconsistent when monolayers received only two overlays. When a second overlay containing 1:20,000 neutral red was added on the 7th day plaques generally did not appear. The evolution of rubella plaques in Vero cells was unusual in that plaques first appeared as hyperstained foci which were surrounded by a zone of relatively destained tissue. These foci gradually became partially destained taking on the appearance of the plaques illustrated in Fig. 4. The incorporation of DEAE dextran in the agar overlay resulted in a 3- to 5-fold increase in plaque count and a less striking but definite increase in plaque size. The efficiency of plaquing is relatively low since plaque titers are 10 times lower than those obtained by CPE endpoint titration in Vero tube cultures.

Primary isolation of virus from clinical specimens. Vero cultures inoculated with serum and throat washings from experimentally infected chimpanzees failed to develop CPE after 21 days of observation. Blind passage of these culture fluids into fresh Vero cultures again resulted in no CPE after 21 days. Similar specimens were inoculated in duplicate into Vero and into pGMK cultures. Fluids were harvested from half of each set of cultures after 11 days and from the remaining half after 21 days. The 11-day fluids were then passed into pGMK and tested for the presence of virus by the interference method. If the 11-day fluid did not contain virus, the corresponding 21-day fluids from the initial cultures were similarly tested

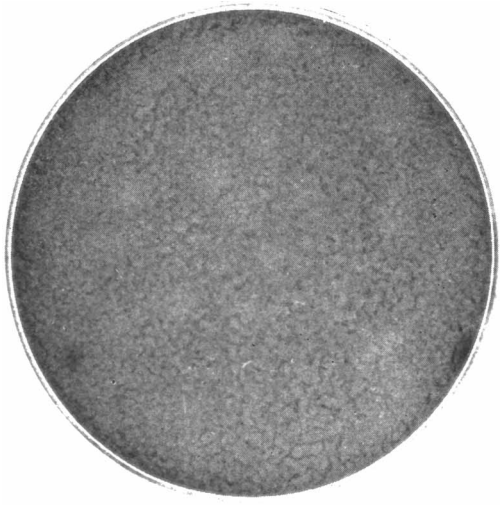

FIG. 4. Rubella virus plaques in Vero monolayer 14 days after inoculation. Magnification ×1.

TABLE II. Comparative Sensitivity of Vero and Primary African Green Monkey Kidney Cells in Primary Isolation of Rubella Virus.

Sequence of isolation procedure		Frequency of positive isolations (No. pos/No. tested)		Overall isolation rate	
Initial culture	2nd passage culture	Serum	Throat swab	No. pos/No. tested	% Pos
pGMK 11d*	pGMK	0/9	3/16	3/25	12
Vero 11d	"	3/9	7/16	10/25	56
Vero 21d†	"	2/6†	2/9 †	4/15	

* Day on which initial culture fluids were harvested for blind passage.
† Specimens which were negative when blind passed after 11 days were blind passed after 21 days.

for the presence of virus. Though unadapted RV failed to produce CPE, it appeared to propagate readily in Vero cultures. Specimens which were initially inoculated into Vero cultures and after 11 days passed into pGMK yielded positive isolations from 10 out of 25 specimens, and if the initial period of culture in Vero cells was extended to 21 days, the total number of specimens yielding virus was increased to 14 out of 25. The isolation rate for the same set of specimens passed twice in pGMK was 3 out of 25. These results are summarized in Table II. In no case was a specimen positive after 2 pGMK passes and negative in the Vero pGMK sequence.

Neutralization tests. A small number of specimens were assayed for their content of neutralizing antibody in Vero cultures by direct neutralization of CPE, and in pGMK cultures by the indirect method of neutralization using interference with echovirus 11 CPE. Neutralizing antibody titers were comparable in both test systems. Although the direct neutralization test in Vero cells required 12 to 14 days before an end point was reached, it was a simpler, more convenient test system than the indirect one.

Effect of various culture conditions on virus growth. Infected Vero cultures, rolled or stationary, maintained in Medium 199, MEM or LHY with 2% or 5% serum at 34° or 37° yielded essentially similar virus titers. Though exact quantitative data are lacking, it was regularly observed that CPE appeared earliest and was more rapidly progressive in cultures maintained in LHY medium with 2% serum.

Complement fixing antigen. Fluids from infected Vero cultures were concentrated 75-100× by dialysis against polyethylene glycol (20,000 MW.) and extracted with ether according to the method of Schmidt and Lennette(19). Fluid harvested from the same monolayer on the 11th and again on the 14th day after inoculation contained specific CF antigen which usually titered 1:8 and rarely 1:16.

Discussion. Methods for detection and quantitation of RV and RV neutralizing antibody have been limited by a number of factors already discussed in the introduction to this paper. Vero cells appear to provide a tissue culture host for RV which is superior in several respects to those previously described. Evaluation of the communicability of attenuated RV vaccines requires more sensitive systems for detection of RV than are now available. It has been shown that a 1:1000 dilution of serum induced rubella when administered to volunteers, yet the same serum at a 1:10 dilution was free of detectable rubella virus in the pGMK echovirus 11 inhibition system(20). On the basis of the data presented here, specimens which were cultured initially in Vero cells followed by one blind passage in pGMK cells yielded virus isolations with 4.5 times greater frequency than they did when passaged twice in pGMK cells alone. A 56% primary isolation rate was achieved with specimens which were cultured by the former procedure, whereas only 12% of the identical specimens carried through 2 successive passages in pGMK cultures alone yielded virus isolations. The primary isolation rate of RV in RK-13(21) and SIRC cells(22) is equivalent to the rate achieved in pGMK cells. Though the RK-13 and SIRC cell

virus isolation procedures are less cumbersome and time consuming, the significantly higher isolation rates yielded by the Vero procedure reported herein fully justify its use.

Vero monolayers were maintained in excellent condition in LHY medium with 2% calf serum with a minimum of non-specific rounding for 2 weeks. Since CPE was easily recognized against the background of a monolayer in good condition, infectivity titrations were read with little difficulty.

With the exception of BHK-21 cells(5), tissue cultures which support the growth of RV do so to relatively low titers. RV titers in excess of $10^{6.5}$ ID_{50}/ml were regularly obtained from Vero cultures. These titers were independent of the input multiplicity of virus in the range from 0.1 to 0.001 ID_{50}/cell. In order to obtain high titered virus from BHK-21 monolayer cultures, a virus multiplicity of 10 must be used. The requirement for a high input multiplicity in BHK-21 cultures was probably necessitated by the rapid degeneration of the monolayer once it had grown to confluence. In contrast confluent Vero monolayers were maintained for periods sufficiently long to allow a small infecting inoculum to undergo many growth cycles. Vero cultures infected with low multiplicities of RV provided an excellent source of complement fixing antigen; the production of RV hemagglutinin is presently being investigated.

Though RV produces plaques in Vero monolayers which are neutralized by rubella antiserum, the assay presents a number of problems which presently limit its usefulness. The factors which are responsible for lack of clarity and the low efficiency of plaquing are currently being investigated. The direct measurement of RV neutralizing antibody in Vero cells was accomplished with little difficulty. The test, however, required 12 to 14 days to complete. A much more rapid and highly quantitative test employing the hemadsorption negative plaque assay was recently reported(23). However, with regard to the need for rapid determination of a patient's immune status or the large scale testing of sera for rubella antibodies, there seems to be little advantage in utilizing any tissue culture system however rapid and simple, since it is now possible to measure RV antibody by hemagglutination inhibition in less than 24 hours.

The growth of RV in Vero cells has been studied independently by Rhim and Schell (24), who have confirmed the observations reported herein.

Summary. Rubella virus has been found to grow to high titer in a continuous line of green monkey kidney cells (Vero), with the production of CPE and plaques. Infectivity titrations can be read within 2 weeks and are somewhat higher than comparable titers measured by echovirus 11 interference in pGMK. Primary isolation rates of 56% were obtained in Vero cultures as compared to 12% with the same specimens tested in pGMK cultures. Complement fixing antigen can be produced in satisfactory titer in Vero cultures, and the system is suitable for the direct measurement of neutralizing antibody.

The competent technical assistance of Miss C. S. Smith is gratefully acknowledged.

1. Weller, T. H., Neva, F. A., Proc. Soc. Exp. Biol. & Med., 1962, v111, 215.

2. McCarthy, K., Taylor-Robinson, C. H., Pillinger, S. E., Lancet, 1963, v2, 593.

3. McCarthy, K., Taylor-Robinson, C. H., Arch. Virusforsch., 1965, v16, 415.

4. Günlap, A., Proc. Soc. Exp. Biol. & Med., 1965, v118, 85.

5. Vaheri, A., Sedwick, W. D., Plotkin, S. A., Maes, R., Virology, 1965, v27, 239.

6. Hull, R. N., Butorac, G., Am. J. Epid., 1966, v83, 509.

7. Leerhøy, J., Science, 1965, v149, 633.

8. Reddick, A., Roesel, C. E., ibid., 1966, v151, 1405.

9. Hok, K., Vaccine Development Board, Rubella Subcommittee Meeting, Jan. 1966, Bethesda, Md.

10. Leerhøy, J., Arch. Virusforsch., 1966, v19, 210.

11. Dudgeon, J. A., Butler, N. R., Plotkin, S. A., Brit. Med. J., 1964, v2, 155.

12. Plotkin, S. A., Arch. Virusforsch., 1965, v16, 423.

13. Parkman, P. D., Buescher, E. L., Artenstein, M. D., Proc. Soc. Exp. Biol. & Med., 1962, v111, 225.

14. Parkman, P. D., Mundon, F. K., McCown, J. M., Buescher, E. L., J. Immunol., 1964, v93, 608.

15. Neva, F. A., Weller, T. H., ibid., 1964, v93, 466.

16. Eagle, H., Science, 1959, v130, 432.
17. Morgan, J. F., Morton, H. J., Parker, R. C., Proc. Soc. Exp. Biol. & Med., 1950, v73, 1.
18. Kärber, G., Arch f exper. Path. u Pharmakol., 1931, v162, 480.
19. Schmidt, N. J., Lennette, E. H., Proc. Soc. Exp. Biol. & Med., 1966, v129. 243.
20. Green, R. H., et al, Amer. J. Dis. Child., 1965, v110, 348.
21. Hutchinson, D. N., Thompson, K. M., Mon. Bull. Min. Hlth. Lab. Service, 1965, v24, 385.
22. Phillips, C. A., Melnick, J. L., Burkhardt, M., Proc. Soc. Exp. Biol. & Med., 1966, v122, 783.
23. Rawls, W. E., Desmyter, J., Melnick, J. L., ibid., 1967, v124, 167.
24. Rhim, J. S., Schell, K., Proc. Soc. Exp. Biol. & Med. 1967, v125, 602.

Received March 13, 1967. P.S.E.B.M., 1967, v125.

An Enzymatic Method to Determine Damage in Human Platelets.* (32168)

NABEEH MOURAD† AND JAMES H. PERT (Introduced by M. B. Zucker)
Blood Program Research Laboratory, The American National Red Cross, Washington, D. C.

Platelet concentrates (PC) are frequently prepared for metabolic and clinical studies by centrifugation of platelet-rich plasma (PRP). This step, and subsequent resuspension of the platelets in about 10 ml of plasma, often causes platelet clumping which may be associated with platelet damage. Acid phosphatase is released from platelets during clotting(1,2), and measurement of released enzymes may possibly provide a quantitative means of assessing platelet damage during preparation of PC. The objectives of this investigation were to find a simple *in vitro* method of detecting platelet damage, and use it to determine whether the degree of damage was affected by the nature of the anticoagulant and, if so, under what conditions it could be minimized.

Methods. Platelet-rich plasma was obtained by centrifuging one unit (about 500 ml) of whole blood containing anticoagulant acid-citrate-dextrose* (PRP-A) or citrate-phosphate-dextrose(3) (PRP-C) in a triple plastic bag (Fenwal Company) at 10°C at 2500 × *g* for 100 seconds, and was expressed into the satellite bag. Experiments were performed on pools of 2 to 4 units of PRP obtained from blood of the same group and type, redivided into equal parts (volume approximately 200 ml). When necessary, acid or adenosine was added to each bag, mixed well, and the pH measured at 10°C. In experiments which included both additives, acid was introduced first, mixed and followed by adenosine. For the preparation of PC, PRP was then centrifuged at 5100 × *g* for 10 minutes at 10°C. The platelets were resuspended in 10-15 ml of the supernatant plasma to obtain PC(4). One ml of the suspension was centrifuged in a plastic tube at 0°C at 35,000 × *g* for 10 minutes. Activity of the enzymes nucleoside diphosphokinase (NDPK), 3-phosphoglycerate kinase (PGK), and enolase was determined in the supernatant so that the amount of enzyme released to the plasma in the PC could be calculated.

Total enzyme content of PRP was determined in lysed platelets. For lysis, 10 ml of PRP was centrifuged at 0°C at 35,000 × *g* for 10 minutes, the plasma was removed and the platelets lysed with 1.35 ml of distilled water. Buffer (0.15 ml of 1.0 M Tris-acetate, pH 7.5) was added, and the lysate was centrifuged at 35,000 × *g* for 10 minutes at 0°C. Enzyme activity was measured in aliquots of the supernatant. We assayed for enzymes which can be measured directly or indirectly by oxidation or reduction of DPN or TPN, and found detectable amounts of the following in the platelet lysates: NDPK, PGK, enolase, lactic dehydrogenase, pyruvate kinase, adenosine triphosphatase, glutathione reductase, hexokinase, glucose-6-

* NIH Formula A.

† Present address: Brooklyn-Cumberland Medical Center, Brooklyn, N. Y., 11201.