tate was fully protective in this regard.

1. Dam, H., Kruse, I., Prange, I., and Sondergaard, E., *Acta Physiol. Scand.*, 1951, v22, 299.

2. Singsen, E. P., Bunnell, R. H., Matterson, L. D., Kozeff, A., and Jungherr, E. L., *Poultry Sci.*, 1955, v34, 262.

3. Dam, H., and Granados, H., *Acta Pharmacol. Toxicol.*, 1952, v8, 47.

4. Johnson, B. C., and Goodyear, S., Third Inter. Congr. on Vit. E, 1955.

5. Ames, S. R., Ludwig, M. I., Swanson, W. J., and Harris, P. L., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 39.

6. Blaxter, K. L., Brown, F., Wood, W. A., and MacDonald, A. M., *Brit. J. Nutrition*, 1953, v7, 337.

7. Draper, H. H., and Johnson, B. C., *J. Animal Sci.*, 1956, v15, 1154.

8. Christensen, F., Dam, H., and Gortner, R. A., *Acta Physiol. Scandinavica*, 1956, v36, 87.

9. Johnson, B. C., Vitamin E, Atti del terzo Congresso Internazionale Venezia 1955, 1956, 456, Edizioni Valdonega, Verona, Italy.

10. Pappenheimer, A. M., *J. Mt. Sinai Hospital*, 1940, v7, 65.

Received April 1, 1957. P.S.E.B.M., 1957, v95.

Adaptation of ECHO Viruses in HeLa Cells; Their Use in Complement Fixation.* (23189)

ITALO ARCHETTI, JEAN WESTON, AND HERBERT A. WENNER
Department of Pediatrics and Hixon Memorial Laboratory, University of Kansas School of Medicine, Kansas City.

The adaptation of ECHO prototype viruses (1) or primary isolation of ECHO viruses in cultures of HeLa cells has not been reported previously. Twelve of the original 13 ECHO viruses have been propagated successfully in cultures of HeLa cells maintained in this laboratory. ECHO virus type 4 failed to grow in HeLa cells.

During preparation of ECHO hyperimmune sera in monkeys, difficulties were encountered in complement fixation (CF) tests with viral antigens derived from infected renal cells. Viral antigens derived from HeLa cells proved to be useful in delineating homotypic antibody, and in exposing serologic cross-relationships observed in neutralization tests. The present report describes the methods used in adaptation of ECHO viruses to HeLa cells, and the application of viral antigens derived from HeLa cells in the CF test.

Materials and methods. Viruses. The prototype ECHO viruses were furnished by J. L. Melnick, Wm. McD. Hammon and A. B. Sabin. We passaged the viruses once or twice in monkey renal epithelial cells(2) when a large pool of stock virus was prepared. The passage history and titers of viruses propagated in cultures of renal and HeLa cells appear in Table I. *Tissue cultures.* The line of HeLa cells was obtained from J. T. Syverton in 1952(3). Since 1955 the cell line has been propagated in yeast extract medium(4) modified as follows: 1% yeastolate (Difco), 8.0%; 20% glucose, 1%; Hank's BSS, 68.0%; 2.8% $NaHCO_3$, 3%; and human serum, 20.0%. Following satisfactory growth in tubes or in bottles the cells were washed 3 times with phosphate buffered saline (PBS). The maintenance solution used in this laboratory(5) consists of mixture 199, 63.5%; 5% $NaHCO_3$, 3%; 20% glucose, 1%; tryptose phosphate broth, 25%; and calf serum, 7.5%. Following addition of maintenance solution cells were ready for inoculation with ECHO prototype viruses. *Virus titrations.* The stock virus pools were titrated in roller tube cultures of monkey kidney epithelial cells. ECHO viruses adapted to HeLa cells were titrated in renal epithelial cell cultures in the colorimetric test originally described by Salk, Youngner, and Ward(6) as modified by Melnick and Opton(7). *Antisera.* Sera were obtained from monkeys (*Macaca mulatta*) hyperimmunized with 13 prototype ECHO

* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Passage Histories, Cytopathogenic Intensities, Antigenic Potencies, and Virus Titers of ECHO Viruses in HeLa Cell Cultures.

ECHO virus	Passage history	Extent of cytopathogenic change in HeLa cultures (%)	Antigenic units, CF test	TCD_{50}/ml* virus derived from HeLa	Renal
Farouk	T_{10} K_9 H_5	90+	2	7.5	7.7
Cornelis	T_9 K_9 H_5	75+	1	6.6	5.8
Morrissey	T_5 K_{12} H_5	50+	1	6.6	7.5
Pesascek	T_9 K_6 H_5	±	1	<1.0	4.5
Noyce	K_8 H_5	75+	2	8.0	8.8
D'Amori	K_{11} H_5	90+	2	6.5	8.4
Wallace	K_6 H_5	90+	1	4.0	8.5
Bryson	K_7 H_5	75+	2	6.7	7.8
Hill	K_{10} H_5	75+	2	7.5	7.2
Lang	K_{13} H_5	75+	8	4.7	7.4
Gregory	K_5 H_5	90+	1	6.0	7.0
Travis	K_- H_5	90+	1	6.8	8.8
Hamphill	K_- H_5	90+	1	5.5	8.1

* Renal-cell-adapted viruses were titrated in renal cell roller tubes. HeLa-cell-adapted viruses were titrated in renal cell cultures in the color test based on decimal dilutions, expressed as log base $_{10}$.

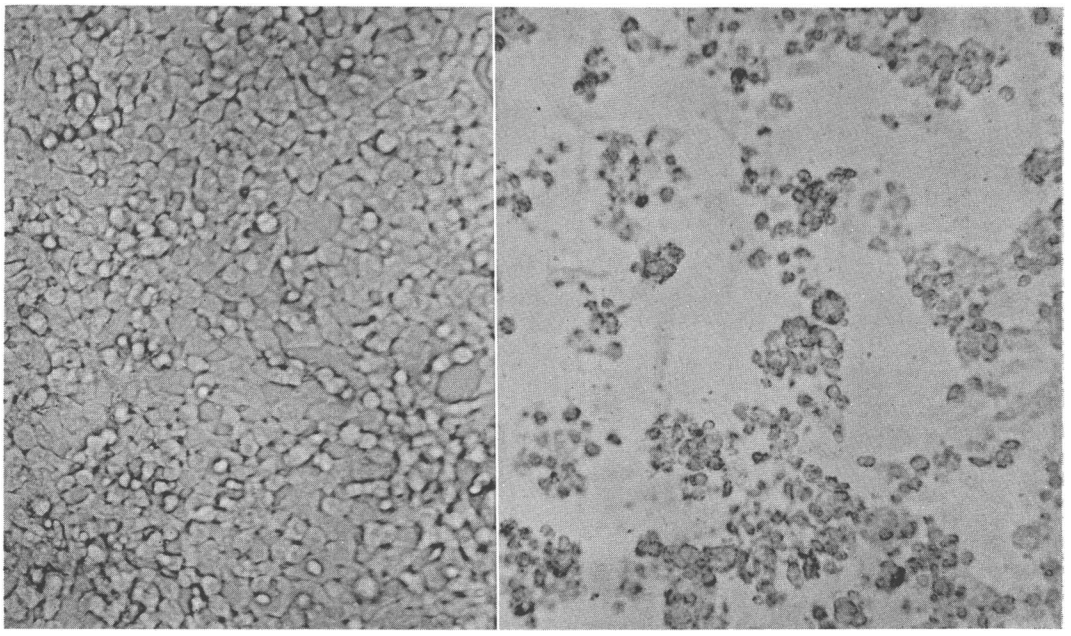
T_{10} K_9 H_5 = 10 passages in testicular fibroblasts, 9 additional passages in kidney cells, and 5 passages in HeLa cell cultures.

viruses.† Pre-vaccinal sera were devoid of homotypic and heterotypic neutralizing antibodies against the 13 ECHO viruses.‡ The immunizing antigens were viruses propagated in monkey renal cells. Each monkey received a series of 5 or 6 intramuscular inoculations. Each inoculum consisted of 1.0 ml of undiluted virus suspended in 1.0 ml of mineral oil adjuvant(8). *CF antigens.* At the 4th passage level in HeLa cells cultured in stationary tubes, milk dilution bottles (200 ml) containing $10^{6.6}$ cells were inoculated with 0.5 ml of each of the 13 ECHO viruses. The volume of maintenance fluid overlaying inoculated cells was 8.0 ml. Cytopathogenic changes were well advanced with all, except type 4 virus, on the 5th day when fluids were harvested. The harvested fluids were treated immediately with $NaHCO_3$ in order to bring the pH to 7.4, and stored in glass-sealed vials at −20°C from whence they were deployed for use in CF tests. *CF tests.* The drop method described by Fulton and Dumbell(9) was adopted with several modifications, most of which have been described previously(10, 11). The concentration of sheep's red cells was 0.5%. The same lot of guinea pig complement was used in the study. Titrations of antibody were done using constant quantities of antigen and complement and doubling dilutions of serum. The unit of complement was taken as the least amount of guinea pig serum that caused complete lysis of the cells. Complement titers were verified by titrations performed coincidental with each test. Antigen, serum, hemolytic, and cell controls were employed. Known negative monkey sera were included in each test. Sera were inactivated at 60°C/30′. Primary incubation was carried out overnight (18 hours) at 4°C. The hemolytic system (1:1000 hemolysin plus 0.5% sheep's rbc) incubated 30 minutes at 37°C was added on the following morning. After a further period of incubation for 2 hours at 37°C the results were recorded. The antibody titer was the highest dilution of serum giving 50% inhibition.

Results. Cytopathogenic action of ECHO

† The following media were used in the propagation of viruses in monkey renal cell cultures which were used to hyperimmunize monkeys: *Growth medium.* Medium D = mixture 199, 95%; horse serum, 2%; and 2.8% $NaHCO_3$, 3%. The growth medium was usually replenished on the 3rd or 4th day. The cells were washed 3 times after which maintenance medium was added. *Maintenance medium* consisted of mixture 199, 97.5% and 5% $NaHCO_3$, 2.5%.

‡ Unpublished data.



a. Normal HeLa cell culture b. Infected with ECHO 9

FIG. 1. Cytopathogenic action of ECHO 9 (Hill) in HeLa cells (unstained preparations 160×).

viruses on HeLa cells. Except for ECHO virus type 4, difficulty in adapting 12 ECHO viruses to cultures of HeLa cells was not encountered. In respect to type 4 cytopathogenic changes were doubtful and were not reproducible, although attempts were made in several serial passages. Furthermore at the 5th passage level measurable amounts of virus were not found on titration in monkey kidney epithelial cells (Table I).

Following primary inoculation of the remaining 12 ECHO viruses in HeLa cells, cellular changes were observed as early as the 3rd day and reached maximal degeneration on the 7th day when most of the cells were visibly affected. On the 3rd day post-inoculation the cells began to separate and had an irregularly rounded appearance. By the 5th or 6th day shrunken misshapen cells had arranged themselves in irregular clusters. Undoubtedly cellular lysis occurred, but the notable persistence of intact altered cells beyond the 7th day suggested that virus multiplication was not necessarily associated with cell destruction.

At each passage level, except the last, the harvested cells were rapidly frozen and thawed 3 times in dry ice and alcohol in order to rupture cells. Such treatment may have had an effect (controlled observations were not made) since in continuous passage there was evidence of adaptation in that fewer clusters of intact abnormal cells remained on glass or in fluid suspension by the 5th day post-inoculation. Despite the progressive effect on cellular lysis several passages by these means were not conducive to complete cellular destruction. The extent of cellular lysis ranged for the several viruses between 70 and 90%. The quantity of virus released in culture fluids ranged between $10^{4.0}$ and $10^{7.5}$ TCD_{50} (Table I). The type of change encountered with ECHO virus type 9 appears in Fig. 1. The changes shown are fairly characteristic of the observed cytopathogenic changes observed with other ECHO viruses propagated successfully in HeLa cell cultures.

Specificity of ECHO virus antigens in the CF test. Viral antigens derived from infected renal cells proved to be anticomplementary and cross-relations between members of the group were encountered in low serum dilutions, (*i.e.*, 1:32 or less). The anticomplementary effect of the antigens was not re-

TABLE II. Specificity of Hyperimmune ECHO Antisera Prepared in Monkeys. Results obtained in CF test.

ECHO virus antigen	Hyperimmune monkey antisera												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Farouk	2048	8	0	0	0	0	0	32	0	0	0	32	128
Cornelis	8	1024	8	8	8	0	0	0	0	0	0	0	0
Morrissey	0	0	1024	0	0	0	8	0	0	0	0	0	0
Pesaseek	—	—	—	—	—	—	—	—	—	—	—	—	—
Noyce	8	8	0	0	2048	0	8	0	0	8	0	0	0
D'Amori	8	0	0	0	0	2048	0	0	0	0	0	0	0
Wallace	0	0	0	0	0	0	256	0	0	0	0	0	0
Bryson	128	8	0	0	0	0	8	1024	0	0	0	16	32
Hill	8	8	0	8	0	0	8	8	512	8	0	8	8
Lang	16	0	0	0	0	0	0	0	0	1024	0	0	0
Gregory	0	0	8	0	0	0	0	0	0	0	512	0	0
Travis	16	8	16	0	8	0	0	0	0	0	0	64	0
Hamphill	1024	0	0	0	0	0	0	256	0	0	0	512	1024
Homologous serum neutralization endpoints* (× 1000)	16	16	25	.2 or <	25	32	16	12	16 or >	16 or >	4.1	20	9

* Against approximately 100 $TCID_{50}$ of homologous virus.
0 = < 1:8 dilution of serum.

moved by heat (56° to 62°C/30'). Moreover, the antigens prepared in renal cells were qualitatively poor in respect to complement-binding capacity. Antigens derived from HeLa cells on the other hand were almost devoid of anticomplementary effect and were qualitatively better, if not entirely ideal, in their application in the CF test.

The results reported here concern use of unheated antigens. Antigens prepared in the manner described had low titers ranging from 1 to 8 units, with an average of 2 units (Table I). Box titrations of antigens did not provide evidence of a zone phenomenon, indicating that minimal rather than excess amounts of antigen were used.

The CF antigens were specific and reliable. The homotypic titers of immune sera prepared in monkeys ranged between 1:64 (type 12) and 1:2048 (types 1, 5, and 6). The results of the tests appear in Table II. The specificity of the CF antibodies was confirmed in neutralization tests. The homotypic neutralizing antibodies are recorded in Table II.

Cross-relationships in the CF test were encountered largely with respect to 3 ECHO viruses, namely types 1, 8, and 13. Each of these viruses reacted also with type 12 antiserum. However, ECHO virus type 12, with the exception of a possible cross with type 1 antiserum, failed to react with type 8 and type 13 antisera. Minor one-directional relationships between ECHO type 10 virus and type 1 antiserum and ECHO type 12 virus and type 3 antiserum remain to be clarified. Except the minor one-directional relationships the results obtained in CF test reaffirmed previous observations on serologic relationships obtained in neutralization tests (Table III).

The cross-relationships encountered above suggested that ECHO viruses types 1, 8, 12, and 13 may share common antigens. However, interpretation on this point should be postponed until there is satisfactory evidence that serologic cross-relationships are reproducible using purified viruses. The chief point of interest concerns the specificity and the reciprocal relationships established in CF and in neutralization tests.

Discussion. The successful propagation of prototype ECHO viruses in HeLa cells was unexpected for the chief reason that primary detection of naturally occurring ECHO viruses in these cells has not come to our at-

TABLE III. Comparison of Cross-Antigenic Relationships Delineated by Neutralization and CF Tests with 4 ECHO Virus Hyperimmune Sera.

	Neutralization endpoints*				CF endpoints			
	1	8	12	13	1	8	12	13
ECHO virus	Farouk	Bryson	Travis	Hamphill	Farouk	Bryson	Travis	Hamphill
Farouk	16,000	500	200	1024	2048	32	32	128
Bryson	1600	12,000	16	100	128	1024	16	32
Travis	<4	<4	20,000	<4	16	<8	64	<8
Hamphill	10,000	160	500	9000	1024	256	512	1024

* Approximately 50 TCD_{50} used; based on avg values of 4 titrations.

tention. Syverton§ has not yet recovered ECHO viruses in HeLa cell cultures. Thus, it may be adduced that HeLa cells are less sensitive than renal cells for detection of ECHO viruses. This, however, is a relative matter, for strains of human origin may prove difficult to detect also in renal cells unless several passages are made. Maximal lytic effect may be observed only after several passages in cultures of renal cells(12). In respect to the adaptation to HeLa cells of virus stocks prepared in renal cells, two possibilities are suggested: namely, (a) that progeny delivered during passage in renal cells contain variants sufficiently represented in the total population to initiate attack of HeLa cells on a scale conducive to visualization, or (b) that the HeLa cell colony continuously in passage in this laboratory has undergone change in respect to susceptibility. The evidence in support of either proposition is insufficient at this time.

Infected fluids harvested from HeLa cells provided specific and reliable although relatively weak antigens in the CF test. ECHO virus type 10 was exceptional and provided a very satisfactory antigen. Heated ECHO viral antigens were less satisfactory than active viral antigens in 2 respects: namely, (a) loss of specificity as indicated by heterotypic cross-reactions with all antisera in low dilution (1:8), and (b) slightly lower homotypic serum dilution endpoints. Sufficient information has not been obtained to establish or to eliminate the presence of soluble antigen.

Better antigens undoubtedly can be prepared. Antigens prepared in cultures of renal cells may prove equal to, or superior to antigens prepared in HeLa cells for diagnoses of human illnesses caused by ECHO viruses. We turned perforce to HeLa cells because of the anti-complementary effect of antigens and antisera derived from monkeys. The cross-reactions observed with antigens derived from monkey renal cells and monkey hyperimmune sera are not entirely understood. The serologic evidence indicates that the cross-reactions were not the result of antibodies to any component in the growth or maintenance media, thereby suggesting interreaction in the monkey to some cellular component released along with virus in culture fluids used to immunize monkeys. Such relationships would not be observed in naturally occurring diseases in human beings or in animals. But whether antigens prepared either in HeLa or in renal cell cultures are sufficiently active for reliable use in measurements of CF antibody response in naturally occurring illness, where neutralizing antibodies remain at levels comparatively lower than in hyperimmunized monkeys, is a subject for additional study.

Summary. Twelve of 13 original ECHO viruses have been adapted to HeLa cells in tissue cultures. The adapted viruses produced discrete, recognizable cytopathogenic changes. Infected fluids at the 5th passage level provided specific, reliable antigens for use in the CF test.

§ Personal communication.

1. Committee on ECHO Viruses, *Science,* 1955, v122, 1187.

2. Salk, J. E., *et al., J.A.M.A.,* 1953, v151, 1081.

3. Syverton, J. T., and Scherer, W. F., *Bact. Proc.,* 1953, 42.

4. Robertson, H. E., Brunner, K. T., and Syverton, J. T., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 119.

5. Ginsberg, H. S., Gold, E., and Jordan, W. S., *ibid.,* 1955, v89, 66.

6. Salk, J. E., Youngner, J. S., and Ward, E. N., *Am. J. Hyg.*, 1954, v60, 214.
7. Melnick, J. L., and Opton, M. E., *Bull. World Health Org.*, 1956, v14, 129.
8. Salk, J. E., Lewis, L. J., Youngner, J. S., and Bennett, B. L., *Am. J. Hyg.*, 1951, v54, 157.
9. Fulton, F., and Dumbell, K. R., *J. Gen. Microbiol.*, 1949, v3, 97.
10. Morgan, P., Carter, J. S., and Wenner, H. A., *J. Lab. and Clin. Med.*, 1953, v42, 665.
11. Svedmyr, A., Enders, J. F., and Holloway, A., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 296.
12. Chin, T. D. Y., Beran, G. W., and Wenner, H. A., *Am. J. Hyg.*

Received April 2, 1957. P.S.E.B.M., 1957, v95.

Enzymatic Release of Pantetheine from Coenzyme A in Animal Tissues And Its Microbiological Determination. (23190)

R. L. WOLFF, S. DUBOST AND J. J. BRIGNON (Introduced by Raymond Cahen)
Laboratoire de Chimie Biologique, Faculté de Médecine, Nancy, France.

Microbiological determination of pantothenic acid(1) or of pantetheine(2) released by enzymatic hydrolysis, has been recommended as a routine method for determination of coenzyme A in animal tissues. The first of these two methods requires the use of 2 enzymes, liver enzyme and intestinal phosphatase. With one enzyme, phosphatase, CoA is split into pantetheine. As the activity coefficient of pantetheine for growth of *Lactobacillus arabinosus* amounts to 20-40% (on a molecular basis) of that of pantothenic acid, microbiological determination of pantetheine, in the presence of preformed free pantothenic acid, becomes very difficult with *L. arabinosus*. With *Lactobacillus helveticus*, another difficulty arises owing to stimulation of the activity of pantetheine by small amounts of pantothenic acid present in the mixture.

Experiments are here reported pointing to a modification of the basal medium for *Lactobacillus arabinosus* 17-5 whereby pantothenic acid and pantetheine, in equimolecular amount, display an identical activity as growth factors. Each of the two factors is devoid of any stimulatory activity towards the other. This method has been checked with the double enzyme method of Novelli and Lipmann.

Method. The basal medium, for *L. arabinosus* 17-5, has been described by Skeggs and Wright(3). It was modified by addition of 50 ml of a 20 mg/ml solution of cysteine hydrochloride to 250 ml of the original medium and was adjusted to pH 6.6-6.8. The tubes were incubated in glass jars at 37°C, for 24 hours in an atmosphere of pure CO_2. *Substrates.* The Ca-pantothenate was of commercial origin. The pantetheine was used as its S-benzoyl derivative,* the identity of the activity for the 2 compounds having been demonstrated. It had been prepared according to Schwyzer(4). *Intestinal phosphatase* was prepared from calf intestinal mucosa according to the first steps of the method described by Schmidt and Thannhauser(5). The trypsin digested mucosa, after filtration, was dialyzed against tap-water for 72 hours and was kept in the freezer at –20°C. *Phosphatase activity* of the solution corresponded to 45 Thannhauser units per ml. Two ml of aqueous tissue extracts, prepared according to the procedure of Kaplan and Lipmann(6), were incubated for 3 hours at 37° with 0.4 ml phosphatase, 0.1 ml 0.12 M $NaHCO_3$ and 47.5 ml H_2O before the assay.

Results. The action of cysteine and CO_2 on growth of *L. arabinosus* in the presence of varying amounts of S-benzoylpantetheine or pantothenic acid is shown in Fig. 1 (I-IV). Cysteine, in the amount of 10 mg per tube of 5 ml, considerably increases activity of pantetheine but not that of pantothenic acid. In an atmosphere saturated with CO_2, activity of either growth factor is slightly increased but that of pantothenic acid to a lesser degree

* Obtained through the courtesy of Dr. Schwyzer, Ciba Inc., Basel, Switzerland.