tively acid resistant. Successful concentration by precipitation at low pH values is, therefore, not surprising. The demonstration that several adenovirus types, including one from a simian source(1), have essentially the same isoelectric point, pH 3.15 ± 0.15, indicates a general similarity in the physical nature and chemical composition of these agents. It will be of interest to learn whether all viruses now included in the adenovirus group have such an isoelectric point and so possibly be established as a taxonomic criterion.

It is evident that the methods described here can be used to concentrate large volumes of adenovirus in a closed, continuous flow system. The technique might be applied, therefore, to problems concerning purification of virus for physical and chemical examination or possibly to concentrate virus from clinical specimens. However, our primary consideration was for preparation of vaccine components. The greatest concentration factor attempted was 100-fold, although considerably greater concentration probably could be achieved either in a primary step or in sequential precipitations and elutions. The extent to which these procedures will be applied to vaccine production is likely to be limited by the practical and economical factors.

Concentration of inactivated vaccine seemed slightly more efficient than that of live virus, but this probably relates to differences in sensitivity of assay methods. Nevertheless, it is tempting to speculate that formalin treatment results in improved aggregation of viral units under optimal isoelectric conditions. This is not unreasonable since other virus preparations, such as influenza or polio, when treated with formalin will sediment more readily in gravitational fields. It is also remotely possible that isoelectric precipitation results in an altered physicochemical state of the inactivated virus so that their antigenicity is increased. Such conclusions, however, must await the development of greater accuracy in titrations of infectivity and antigenic potency.

Summary and conclusions. The isoelectric optimum of 6 types of human adenoviruses in undialyzed monkey kidney harvest fluids has been established as pH 3.15 ± 0.15. Live and formalin-inactivated viruses were concentrated efficiently by elution of isoelectric precipitates from Celite columns. These observations appear to have several practical applications, as in the preparation of more potent vaccines.

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Production of Hemagglutinin by Dengue Virus in HeLa Cells. (28343)

SONJA M. BUCKLEY AND SUNTHORN SRIHONGSE* (Introduced by J. Casals)
The Rockefeller Foundation Virus Laboratories, New York City

Attempts at demonstrating *in vitro* formation of hemagglutinins by arthropod-borne viruses have been numerous(1,2,3,4,5), both in cultures of stable cell lines and in primary tissue cultures. Dengue virus, however, has been absent from the list of agents thus characterized.

In this laboratory, dengue virus has been adapted to HeLa cells with concomitant cytopathic effect (CPE)(6). In the course of the further studies reported here, representative strains of types 1, 2, 3 and 4 have been passed serially over a period of 2 years, and

* International Postdoctoral Research Fellow of Nat. Inst. Health.

positive tissue culture hemagglutination-hemadsorption (THA) tests(7) have been obtained regularly with high passage levels of types 1, 2 and 3 and sporadically with type 4. As a consequence of this suggestive evidence of the presence of hemagglutinin (HA) in the fluid phase of infected HeLa cell cultures, *in vitro* production of HA by dengue virus has been studied with special reference to possible differences in HA titers produced by unadapted mouse brain virus and HeLa cell adapted virus.

Materials and methods. Virus. The following dengue strains were employed: type 1, Hawaiian strain, 78th mouse brain passage and 53rd HeLa cell passage; type 2, New Guinea strain, 52nd mouse brain passage and 62nd HeLa cell passage; type 3, strain H-87, 28th mouse brain passage and 53rd HeLa cell passage; type 4, strain H-241, 12th mouse brain passage and 43rd HeLa cell passage.

Cell cultures. HeLa cells were grown in 160-ml French square bottles in a growth-promoting medium consisting of 30% human adult serum, 10% fetal bovine serum, 1% vitamin stock solution (100 × concentrated),† 44% Hanks' balanced salt solution and 15% tryptose phosphate broth (Difco), supplemented with 100 units of penicillin, 100 μg of streptomycin and 25 μg of mycostatin per ml. The pH of the growth-promoting medium was 7.4 ± 0.1. The cell inoculum per stock bottle was 10 ml of a cell suspension containing 50,000 cells per ml. Cells in stock bottles were fed twice weekly, and cellular transfers were carried out regularly at weekly intervals. For cultures of the slant-tube type (150 × 16 mm), trypsin-dispersed cells, 100,000 per ml, were suspended in an outgrowth medium consisting of 30% human adult serum and 70% Eagle's basal medium supplemented with 100 units of penicillin and 100 μg of streptomycin. The pH was 7.2 ± 0.1. Cell cultures were inoculated after an initial incubation period of 72 hours at 37°C.

Inoculation of tubes. The outgrowth medium was poured off and the cell sheets were washed with Hanks' solution, which was then replaced with 0.9 ml of maintenance medium composed of 97% Eagle's basal medium and 3% fetal bovine serum supplemented with 100 units of penicillin and 100 μg of streptomycin. The pH of the maintenance medium was adjusted to 7.5 ± 0.1 with Tris(hydroxymethyl) aminomethane, which was prepared as a 2 M solution in demineralized water, filtered through a Seitz pad and added in amounts of 0.25 ml per 100 ml of medium. Virus dilutions were prepared with a diluent consisting of 0.75% bovine plasma albumin in phosphate buffered saline at pH 7.2, and inoculated in 0.1-ml amounts. Inoculations were carried out at the beginning of the week. Fluids were changed daily during the first 4 days following cellular infection (1 ml), thereafter at 2- to 3-day intervals (2 ml). Cultures were examined at frequent intervals for 21 days.

Titration of virus. Increasing 10-fold dilutions of virus were inoculated into newborn mice and HeLa cell cultures. Cultures were examined for development of CPE and THA for 21 days following inoculation. Virus titers were expressed as LD_{50}, TCD_{50} and $THAD_{50}$ per ml.

Tissue culture hemagglutination-hemadsorption (THA) test. This test is indicative for the presence or absence of cell-associated viral HA(7). Cultures to be tested were placed in a stainless steel tissue culture rack.‡ Fluids of infected and uninfected cultures were removed, and the cellular monolayer of each tube was washed once with 1 ml of Hanks' balanced salt solution adjusted to pH 7.4-7.6. The washing fluid was discarded, and borate saline (pH 9.0)(8) was added, 0.2 ml per culture. The cultures were then incubated in a horizontal position at 37°C for 60 minutes, so that the borate saline overlaid the cells. Goose erythrocytes suspended in virus-adjusting diluent(8) to give a final pH of 6.6 (for dengue types 1, 2 and 4) and 6.5 (for dengue type 3) were then introduced, 0.2 ml per culture, and mixed thoroughly with the borate saline. The optical density of the red cell suspension corresponded roughly to a dilution of 1:11 of the standardized cell stock(8). The cultures were then

† Microbiological Associates, Bethesda, Md.

‡ The Seelye Craftsmen, Minneapolis, Minn.

TABLE I. Adaptation of Dengue Virus to HeLa Cells.

Dengue type	Passage	Onset of CPE (days)	TCD_{50}/ml (neg $\log_{10}$)	$THAD_{50}$/ml (neg $\log_{10}$)	LD_{50}/ml (neg $\log_{10}$)
1	78, mouse brain	13	3.2	0	8.6
	53, HeLa	3	7.5	7.5	6.8
2	52, mouse brain	2	9.5	9.5	8.8
	62, HeLa	3	6.7	6.7	6.2
3	28, mouse brain	4	8.7	8.7	8.6
	53, HeLa	3	7.2	7.2	5.9
4	12, mouse brain	5	4.5	0	7.2
	43, HeLa	4	4.7	0	5.9

incubated in a horizontal position at room temperature for 2 hours, during which time the goose erythrocytes overlaid the HeLa cells under pH conditions optimal for hemagglutination of dengue virus. The tissue culture rack containing the cultures was then rotated so that the tube walls free of HeLa cells faced the bench table. Tubes were allowed to drain in this position at an angle of 10° for 5 minutes, after which they were left for 10 minutes in a vertical position, extreme care being taken not to agitate the cultures in any way at this stage of the test. The tests were then read immediately under a light microscope (100 ×). As described previously(7), test readings were based exclusively on chain formation of goose erythrocytes. Results were scored as follows: +, chain formation (3 or more erythrocytes); ±, occasional chains (2-3 erythrocytes); 0, no chain formation. In all tests, uninoculated controls were completely free of chain formation.

Preparation and titration of hemagglutinating antigens. Aliquots of infected fluids were collected in a pre-chilled tube and stored in a container with crushed ice. With a pre-chilled syringe fitted with a 25-gauge needle, fluids were added drop by drop, with shaking, to a 50-ml pre-chilled centrifuge tube containing 20 ml of cold acetone. Extraction was carried out for 5 minutes in the ice bath, with periodic shaking, and the material was then centrifuged at 1,000 rpm for 5 minutes in the cold. After most of the supernatant acetone had been aspirated off, the precipitate was reextracted for 30 minutes with the same volume of chilled acetone in a stoppered tube, vigorous shaking being necessary to resuspend the pellet. After recentrifugation at 1,000 rpm for 5 minutes and removal of the bulk of the acetone, the extracted tissue culture material was dried at room temperature with a water pump and dissolved in a measured amount of borate saline, pH 9.0(8) to give a 4-fold concentration of the original volume. Titrations of hemagglutinating antigens in plastic plates were performed by techniques described in detail by Clarke and Casals(8). Before titration, rehydrated material was stored either at 4°C for 18 hours or at room temperature for 2 hours. After addition of the goose erythrocytes(8), the plastic plates were incubated at room temperature for 1 hour. The titration endpoint was taken as that dilution of hemagglutinating antigen showing complete hemagglutination of goose erythrocytes. Optimal pH values, determined at room temperature, were 6.6 for dengue types 1, 2 and 4 and 6.5 for dengue type 3.

Results. Adaptation of dengue virus to HeLa cells. Table I summarizes the results obtained with the 4 dengue types. Types 2 and 3 clearly behaved differently from types 1 and 4. Small doses of types 2 and 3 unadapted mouse brain virus caused clear-cut CPE, so that the correlation was close between TCD_{50} and LD_{50} titers of the inoculum. Furthermore, both types gave a positive THA, at pH 6.6 for type 2 and at pH 6.5 for type 3; $THAD_{50}$ titers were identical with TCD_{50} titers. By contrast, small doses of types 1 and 4 unadapted mouse brain virus killed newborn mice but failed to induce clear-cut CPE in HeLa cells; this explains the discrepancy between the TCD_{50} and LD_{50} titers of the inoculum. THA was sporadically positive with type 1 mouse brain virus but negative with type 4. After prolonged *in vitro* cultivation, however, small doses of types 1

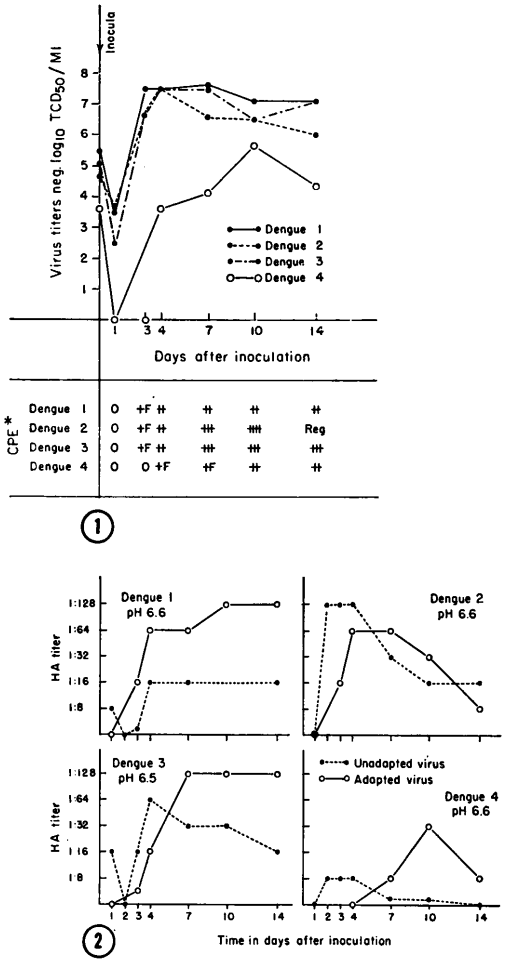

FIG. 1. Comparison of growth curves of high passage level HeLa cell adapted dengue virus types 1, 2, 3 and 4.

* Legends—CPE: 0, no cellular changes; +F, multiple focal lesions; ++, 25% cells detached from glass, other cells rounded; +++, 50% cells detached from glass, other cells rounded; ++++, 90% cells detached from glass, other cells rounded; Reg, regeneration of cells.

FIG. 2. HA titer per ml fluid phase; concentration factor = 4. ●----● Unadapted mouse brain virus; ○——○ High passage level HeLa cell adapted virus.

and 4 HeLa cell adapted virus induced clear-cut CPE; THA was consistently positive with type 1 at pH 6.6 and occasionally positive with type 4 at this pH.

Changing the maintenance medium daily during the first 4 days after inoculation shortened the incubation period to the extent that all 4 types induced CPE within 3-4 days following inoculation of undiluted tissue culture fluid. Growth curves obtained with high passage level virus are illustrated in Fig. 1. With types 1, 2 and 3 peak TCD_{50} values were reached within the first 4 days following infection. With type 4, the logarithmic phase of the growth curve occurred 3-4 days post-inoculation, and the phase of retardation from 4-10 days following infection.

Production of HA by unadapted mouse brain virus and high passage level HeLa cell adapted virus. Fluids from cell cultures inoculated with a 10^{-2} dilution of either unadapted mouse brain virus or high passage level HeLa cell adapted virus were tested for the presence of HA at 1, 2, 3, 4, 7, 10 and 14 days postinoculation. Optimal pH values of 6.6 for types 1, 2 and 4 and 6.5 for type 3 corresponded with the values found optimal in the THA test. The results of these experiments are presented in Fig. 2.

HA titers of 1:128 and 1:64 were obtained with types 2 and 3 unadapted mouse brain virus, whereas types 1 and 4 unadapted mouse brain virus, despite high infectivity titers in newborn mice, gave HA titers of only 1:16 and 1:8. HA titers reached a peak early, within 2-4 days following inoculation; during the second week after inoculation, titers declined with types 2, 3 and 4 and remained stationary for type 1.

When high passage level HeLa cell adapted virus was inoculated, HA titers were increased 8-fold for type 1, 2-fold for type 3 and 4-fold for type 4. Titers reached a peak within 4 days postinoculation for types 1 and 2, 7 days for type 3 and 10 days for type 4. Under the experimental conditions, type 2 HeLa cell adapted virus was distinct from the others and had a lower peak of HA titer than type 2 mouse brain virus.

Discussion. Diercks *et al.*(4) reported that dengue types 1 and 2 neither propagated nor produced HA in primary hamster kidney tissue cultures. Salminen(5), in spite of using an inhibitor-free maintenance medium containing bovine plasma albumin instead of serum, was unable to obtain HA from human amnion cells of a stable line infected with dengue type 2, although infective virus was present in the cell cultures. The same author

failed to demonstrate HA in human amnion cells infected with Chikungunya virus. Since both dengue type 2 and Chikungunya(9) produce HA in our HeLa cell line, the different results obtained in the two laboratories may derive from a difference in the susceptibility of the two cell lines or from a difference in maintenance conditions of the cell cultures during the period of viral synthesis. Likar(10), studying factors influencing the development of HA, came to the conclusion that the low percentage (3%) of serum in the maintenance medium had no demonstrable effect on the HA titer in fluids of infected cell cultures, either before or after acetone extraction.

Adaptation of dengue virus to HeLa cells has been facilitated by the observation that daily fluid changes of infected cell cultures during the period of viral synthesis shortened the *in vitro* incubation period, rendered CPE more clear-cut and increased the TCD_{50} titers. Hallauer(11) has described the procedure of daily fluid changes in his studies on *in vitro* propagation of yellow fever virus. It is assumed that frequent renewal of the maintenance medium renders the noninfected cells highly susceptible to infection, while at the same time removing any interferon or antiviral substance produced by the cells. In his study, Likar(10) found that frequent changes of the maintenance medium did not interfere in the least with production of HA. The beneficial effect of this rather laborious procedure is clearly illustrated in Fig. 1, which shows that the logarithmic phase of the growth curves of high passage levels of the 4 dengue types occurs between 1 and 4 days following infection.

In the experiments reported here, *in vitro* adaptation favored the production of HA for dengue types 1, 3 and 4. Sufficient *in vitro* multiplication of dengue virus appears to be the most important factor in *in vitro* production of HA.

Recently, ultrafiltration of infected fluids prior to acetone extraction has been used by Likar *et al.*(12), with concomitant marked increase in HA titer. Infected fluids were concentrated up to 100 times with an ultrafilter, LKB 6300 A,§ a unit frequently used for concentrating aqueous solutions of substances with high molecular weights. It is hoped that by this means a significant increase in HA titer of fluids of cell cultures infected with dengue virus may be realized.

Summary. Production of hemagglutinin by dengue virus types 1, 2, 3 and 4 has been demonstrated in the fluid phase of HeLa cells infected either with unadapted mouse brain virus or with high passage level HeLa cell adapted virus.

Cell cultures inoculated with high passage levels of HeLa cell adapted virus of all 4 types showed the presence of hemagglutinin in infected fluids at the time of the first appearance of the cytopathic effect. High titers of hemagglutinin correlated with high TCD_{50} titers.

Adaptation to *in vitro* conditions favored the production of hemagglutinin in cell cultures inoculated with dengue types 1, 3 and 4. Under the experimental conditions, type 2 unadapted mouse brain virus yielded higher concentrations of hemagglutinin than did type 2 high passage level HeLa cell adapted virus.

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