extensive N-demethylation of levorphanol (20%) as estimated by $C^{14}O_2$ elimination as compared with a limited ability to N-demethylate morphine (ca. 1%). The dog on the other hand appears to have little ability to N-demethylate either levorphanol or morphine. It is interesting that a similar pattern of O-demethylation of codeine obtains in the dog and monkey, *i.e.*, the monkey has a moderate capacity (8.0% of the dose) for O-demethylation while the dog has little or no capacity for this function (Woods *et al.*(8)).

It is apparent, therefore, that small changes in chemical configuration are capable of extensive modification of the metabolic pattern of the potent analgesics.

Summary. The results of urinary excretion and $C^{14}O_2$ elimination experiments in dogs and monkeys receiving 2.0 mg/kg of N-C^{14}-methyl morphine, subcutaneously, were as follows: dogs excreted 13-15% of total dose as free morphine and 48-52% as conjugated morphine and about 0.2% as exhaled $C^{14}O_2$. Monkeys excreted 6-14% as free morphine and 55-71% as conjugated morphine and about 1% as exhaled $C^{14}O_2$.

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Growth of Certain Arthropod-Borne Viruses in Hamster Kidney Tissue Culture. (26294)

CHARLES R. ROSENBERGER AND CHARLES W. SHAW (Introduced by K. Habel)
Department of H. E. W., Public Health Service, Nat. Inst. of Health, Nat. Inst. of Allergy and Infectious Diseases, Laboratory of Tropical Virology, Bethesda, Md.

In recent years investigators have shown successful propagation of selected arthropod-borne viruses in various tissue culture systems(1-5) including hamster kidney tissue culture (HKTC). A single tissue culture system sensitive to many of the arthropod-borne viruses is particularly desirable. In our laboratory a long term study is being made to evaluate tissue cultures as host systems for isolation of viruses, study of virus cell relationships, and production of viral reagents. It has been our experience that hamster kidney (HK) cells are outstanding among the many cell lines under investigation in serving as a culture medium for an increasing number of viruses.

To evaluate HKTC as a suitable standard cell system for arthropod-borne viruses in respect to cytopathogenic effect (CPE), sensitivity, and viral growth, the present studies were made with 4 group A viruses: eastern equine encephalomyelitis (EEE), western equine encephalomyelitis (WEE), Sindbis, and Mayaro; and 5 group B viruses: Ilheus, St. Louis encephalitis (SLE), Japanese encephalitis (JE), Murray Valley encephalitis (MVE), and West Nile (WN).

Methods. Tissue cultures (TC) were prepared essentially according to Youngner's (6) technic. After 5-15 days, cultures containing full cell sheets and 1.5 ml of maintenance medium(7), were inoculated with 0.3

ml of a 10^{-3} dilution of virus material. Passes were made from inoculated cultures by transferring 0.3 ml of the fluid phase to fresh monolayer HKTC. Passes in TC were made according to viral activity indicated by CPE, and the last 2 passes were made during the period of greatest viral concentration previously determined by growth curves. In a few instances the fluid phase containing virus was frozen and stored at –70°C until inoculated into mice or TC. No material was stored longer than 10 days.

Comparative titrations were made using the same serial 10-fold dilutions and inoculating 0.03 ml intracerebrally into each of 5 mice per dilution and 0.3 ml into each of 5 TC tubes per dilution. Tissue cultures were observed 7 days, and those showing CPE were recorded as positive. Mice which died within a 2-14 day observation period were recorded as infected with virus. Samples taken for growth and inactivation curves were considered undiluted material from which 10-fold dilutions were made. No adjustment in titer was made to compensate for the greater inoculum in TC.

The viruses used for the initial passage in TC and for growth and inactivation curves were from infected mouse brain suspensions which had been stored in sealed glass ampoules in a dry ice box. Six of the viruses (EEE, WEE, WN, SLE, JE, and MVE) used in this study were obtained from the American Type Culture Collection, Washington, D. C., and are described in the Viral and Rickettsial Registry(8). Sindbis(9) and Mayaro(10) viruses were obtained from Dr. Theiler of Rockefeller Inst. for Medical Research. Ilheus(11) virus was supplied by Dr. McCrumb of Univ. of Maryland.

Rate of viral growth was estimated by titration of the fluid phase from inoculated HKTC after various periods of incubation. Titrations of group A viruses were made in TC; other viruses were titrated in mice. Cultures having a full monolayer cell sheet were inoculated with 0.3 ml of a 10^{-2} dilution of virus. A sample of the fluid from 2 tubes was then immediately pooled and frozen until titrated. Inoculated tubes were incubated at 35°C for 30-45 minutes to allow time for possible adsorption of virus. The excess inoculum was then removed and cultures were washed 4 times with 1 ml volumes of TC maintenance medium to remove unadsorbed virus. The last wash was saved for titration and on charts represents the 0 hr sample. The cultures were then replenished with 1.5 ml of maintenance medium, and incubation at 35°C continued. At 4 or 12 hour intervals during a 7-day period 2 tubes were selected at random and the supernatant fluid pooled and titrated.

Data for plotting inactivation curves were obtained by titration of virus at intervals during incubation at 35°C in TC maintenance medium without cells. Tubes containing 1.5 ml of medium were inoculated with virus and the fluid from 2 of these tubes pooled, frozen, and stored at –70°C. Subsequent samples were taken by pooling and freezing the fluid from 2 additional tubes at each sampling time during a 10-day period. Titrations of the stored frozen samples were made in 10-12 g mice.

After 10-12 passages of virus in TC, dilutions of each virus were tested for neutralization by a constant concentration of specific homologous antiserum previously shown to have a neutralization index of 2.0 or greater in mice. Antisera were prepared by repeatedly inoculating rabbits with mouse brain suspensions of virus which had not previously been passed in TC.

Results. The viruses used in this study each caused a CPE which was reproducible through 10-15 passages in HKTC with only slight variation in intensity and time required to cause observable changes in cells. The most variable results were shown by MVE virus which caused less CPE than the other viruses studied. It was found that these viruses could be grouped into 3 categories according to time required to cause CPE. The group A viruses: EEE, WEE, Sindbis, and Mayaro produced the most marked CPE, causing complete destruction of cells in 48 hours or less. The second category included the group B viruses: SLE, JE, MVE, and WN and gave marked CPE,

TABLE I. Log LD_{50} Titer in Mice and Tissue Culture of Arthropod-Borne Viruses after Passage in Mouse Brain.

Virus	No. MB passages	Titer in mice (per .03 ml)	Titer in HKTC (per 0.3 ml)
EEE	460	8.2	9.4
WEE	25	8.3	8.4
Sindbis	5	5.8	5.3
Mayaro	10	6.6	7.0
Ilheus	28-35	8.5	8.0
SLE	107	7.8	8.0
JE	45	8.7	7.5
MVE	12	7.6	8.5
WN	30	7.4	6.6

usually progressing to 75% cell degeneration after 3-5 days incubation. The CPE caused by Ilheus virus was different in that it usually involved 75% of the cells within 48-56 hours but did not consistently affect the entire cell sheet. It was found that the CPE produced by JE virus was increased in intensity by rapid passages of unfrozen virus laden TC fluid in fresh HK cells so that complete cell degeneration occurred within 24-36 hours after inoculation. This may have been caused by increase of virus in the inoculum although it did not occur with the other viruses.

The titer in TC of mouse brain virus material did not differ widely from its titer in mice (Table I). Control TC inoculated with a suspension of normal mouse brain did not show cell degeneration.

After 10 or more passages in TC marked loss of pathogenicity for mice was noted for each of the viruses as evidenced by much higher titers in TC than in mice (Table II). Exceptionally low titers in mice, as compared to titers in TC, were obtained with Ilheus, SLE, and MVE viruses.

TABLE II. Log LD_{50} Titer in Mice and Tissue Culture of Arthropod-Borne Viruses after Passage in Hamster Kidney Tissue Cultures.

Virus	No. MB passages	No. TC passages	Titer in mice (per .03 ml)	Titer in HKTC (per .3 ml)
EEE	460	15	5.2	7.5
WEE	25	11	4.7	7.2
Sindbis	5	11	6.0	7.5
Mayaro	10	11	5.8	6.6
Ilheus	28-35	12	2.8	7.3
SLE	107	12	2.5	7.0
JE	45	12	4.8	7.3
MVE	12	12	2.3	7.4
WN	30	12	4.8	7.8

Growth of group A viruses was very rapid. Eastern equine encephalomyelitis virus attained the greatest concentration in the fluid phase of TC 16 hours after inoculation when it had increased in titer from 2.6 log $TCID_{50}$ at 0 hours to 9.4 log $TCID_{50}$, while peak growth of WEE, Sindbis, and Mayaro viruses occurred within 24-48 hours (Fig. 1). Group A viruses increased considerably more in titer than Group B's with the exception of Ilheus virus which increased in titer from 1.0 to 7.7 log $TCID_{50}$ with 75% CPE in 56 hours (Fig. 1, 2, 3). The CPE caused by the group A viruses had affected about 50% of the cell sheet at time of greatest viral concentration whereas the CPE caused by group B viruses (SLE, JE, MVE, and WN) involved 25% of the cell sheet when highest titers occurred.

Titrations of samples of virus taken during inactivation resulted in the rates of inactivation shown in Fig. 1, 2, and 3. It was found that the viruses were essentially inactivated after 5 days at 35°C in TC maintenance medium. It is possible that the concentration of virus in the initial suspension may have had some effect on rate of drop in titer. The group B viruses did not persist so long in cell free media, being completely inactivated after 3 days while with group A viruses trace amounts of virus could still be detected at 5 days.

Neutralization tests of the TC passage material in TC resulted in neutralization of 2-5 logs of virus with the exception of SLE virus. The SLE tissue culture material was not neutralized in mice or TC unless it was first passed twice in mice. It was then neutralized in mice but no CPE was prevented in TC.

Discussion. The results presented here indicate that HK cells are particularly suitable as a culture medium for many arthropod-borne viruses. Since the viruses used in this study were all mouse adapted strains, the results are not necessarily applicable to viruses isolated from naturally infected hosts. However, these results provide an indication of probable usefulness of HKTC in isolation

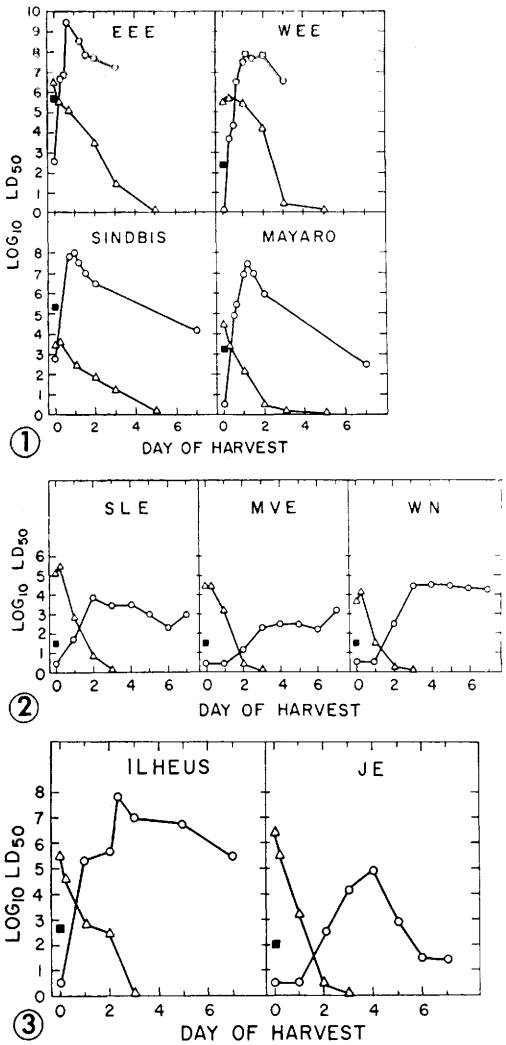

FIG. 1. Growth in hamster kidney tissue culture and inactivation in tissue culture medium without cells of eastern equine encephalomyelitis, western equine encephalomyelitis, Sindbis, and Mayaro viruses.

FIG. 2. Growth in hamster kidney tissue culture and inactivation in tissue culture medium without cells of St. Louis encephalitis, Murray Valley encephalitis, and West Nile viruses.

FIG. 3. Growth in hamster kidney tissue culture and inactivation in tissue culture medium without cells of Ilheus and Japanese encephalitis viruses.

○ = growth; △ = inactivation; ■ = amount of virus in growth curve cultures before washing to remove unadsorbed virus.

work. Isolation of a type 2 dengue virus in HKTC has been reported(12), and Schmidt *et al.*(13) found HK cells to be the most sensitive of 7 cell lines tested when working with newly isolated Uruma virus. In the present work HK cells were equally sensitive to either mouse adapted or TC adapted strains of virus whereas mice were more sensitive to mouse adapted virus so that HKTC might detect variants of arthropod-borne viruses which would be avirulent in mice.

After the work presented had been completed, a contaminating organism was isolated from apparently normal HK cell lots. This contaminant was suppressed by antibiotics in the TC medium so that it was undetectable until increased by passage, causing a virus-like CPE in HK cells. It could not be grown in bacteriological media until passed 2 or 3 times in TC without antibiotics. The contaminant is tentatively referred to as "hamster agent" and appears to be a bacterial L form.

It occurred to us that the TC passages used in this work may also have contained the "hamster agent." When the last TC passage of each of the viruses was checked, it was found that all except EEE were contaminated with this organism. However this "hamster agent" although present was not responsible for the CPE in titrations of the various viruses since all but SLE were neutralized in TC by antisera which had been prepared against mouse brain viral antigen. In the case of SLE it was shown that the TC titer end-point was due to this "hamster agent" thus explaining the lack of neutralization with anti-SLE serum when tested in TC.

That the presence of the "hamster agent" did not influence virus growth was shown by equal virus titers when the virus was propagated in agent contaminated and uncontaminated cultures.

The significance of the contaminant is not presently known, but its previously undetected presence, the virus-like CPE, and the fact that it may be present in hamsters may be of considerable importance whenever hamster cell cultures are used in virus work.

Summary. An investigation of certain group A and B arthropod-borne viruses in HKTC indicated that in general the group A viruses gave greater CPE and increased in titer more rapidly than the group B viruses studied. Comparative titrations in mice and TC were approximately equal for mouse

adapted strains of virus, but after passage in TC, all the viruses showed some loss of pathogenicity for mice.

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A Biochemical Comparison of 6-Aminopenicillanic Acid, Benzylpenicillin, and 2,6-Dimethoxyphenylpenicillin. (26295)

HARRY G. STEINMAN
Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

With the advent of many new semi-synthetic penicillins as the result of the availability of 6-aminopenicillanic acid(1) as precursor material, it will be possible to examine in greater detail the problem of correlation of chemical structure of the penicillins with their biological and biochemical activities. In this paper one of the newer penicillin derivatives, 2,6-dimethoxyphenylpenicillin, which has shown promise in treatment of penicillin-resistant staphylococcal infections(2,3), benzylpenicillin, and 6-aminopenicillanic acid are compared as substrates for penicillinase, as growth-inhibitory agents, as inducers of penicillinase, and as selectors of penicillin-resistant mutants.

Materials and methods. 2,6-Dimethoxyphenylpenicillin (DMP)* was supplied by Bristol Laboratories, benzylpenicillin (penicillin G) by Squibb Research Inst., and 6-aminopenicillanic acid (6-APA) by Pfizer Research Laboratories. *Hydrolysis* of penicillin by penicillinase was measured manometrically in the Warburg apparatus through liberation of CO_2 from a $NaHCO_3$ buffer system by formation of penicilloic acid(4). The enzymes used were cell-free preparations of penicillinase obtained from *Bacillus cereus* and from *Staphylococcus aureus*, which were partially purified and concentrated by isoelectric precipitation(5). *Minimum* growth-inhibitory concentration of penicillin was determined by turbidimetric observations, over a 5-hour period, of the rate of growth of cultures in trypticase soy broth(6). The cultures were shaken in an air-bath (New Brunswick Gyrotory) at 37°C in 300 ml Erlenmeyer flasks equipped with side-attached test tubes for optical density measurements (Bellco Glass, Inc.). The test organisms were penicillinase-producing strains of *B. cereus* (ATCC 10876; NRRL B-569) and *S. aureus*(7). Induced formation of penicillinase by *B. cereus* and by *S. aureus* was carried out as described previously(7). *Selection* of strains resistant to penicillin was accomplished by serial passage of the bacteria through media containing increasingly higher concentrations of the antibiotic(8). Both penicillin-sensitive and penicillin-resis-

* 6-(2,6-dimethoxybenzamido)penicillanic acid (Staphcillin, Celbenin).