termediate salt content. At high concentrations of calcium (275 ppm) this difference is eliminated and the ratio becomes the same.

On the other hand, experiments with strontium-90 indicate that the uptake of this element by the body and the spine:body ratios are similar for both strains of fish. It is interesting to note that the strontium-90 spine:body ratio for both strains of fish is similar to the calcium-45 spine:body ratio of lordotic animals. We have previously shown that the proportion of strontium-90 incorporated into bone, relative to the rate of uptake by the total carcass of wild-type guppies is significantly greater than for calcium-45(9). The significance of these data is obscure at the present time and must await further clarification.

These data demonstrate that the hereditary mutation resulting in lordosis is associated with a specific metabolic abnormality involving calcium metabolism. The mutant guppy, easily raised in large numbers under laboratory conditions, may aid in the elucidation of calcium metabolism and bone formation in vertebrate animals, but a complete understanding of this mutant fish must await further, more definitive studies.

Summary. The lordotic mutation in the guppy is associated with a lower accumulation of body calcium and increased incorporation of calcium into bone. Strontium incorporation is the same for both strains of fishes. These studies indicate a specific defect in calcium metabolism which may further an understanding of calcium metabolism and bone formation.

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Hamster Kidney Cell Tissue Cultures for Propagation of Japanese B Encephalitis Virus.* (23827)

FRED H. DIERCKS AND WILLIAM McD. HAMMON
Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh

Since 1949, there has been much developmental laboratory work done with monkey kidney and HeLa cell tissue cultures and the more stable viral agents such as polio, Adeno, and ECHO groups. However, these cell systems frequently have failed to yield marked and consistent cytopathogenic effects (CPE) following inoculation with more labile agents as exemplified by arthropod-borne group. Consequently, the search for new tissue culture systems to permit an *in vitro* methodology for these less stable viruses is continuing. The earlier work has been admirably reviewed by Sanders, Kiem and Lagunoff(1). Syverton and Scherer(2) reported serial propagation of Western (WEE), Eastern (EEE), West Nile (WN), St. Louis (SLE), and Japanese B (JBE) encephalitis viruses in HeLa cell tissue cultures noting irregular and inconsistent CPE with SLE and JBE agents. McCollum and Foley(3) reported successful propagation of JBE virus in monkey kidney, chick fibroblasts, and Detroit-6 cell line tissue cultures, but without complete CPE. Banta(4) reported attempts to cultivate JBE, WEE, WN, and dengue (type

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1) agents in a variety of cell lines and consistent with Scherer's findings, observed CPE of WEE and WN in HeLa cells and in addition, in Henle's human intestinal cells. His infectivity tests revealed evidence of multiplication of all 4 viruses in monkey kidney cells. Bhatt and Work(5) using monkey kidney and chick embryo tissue cultures to propagate WN, Tamil Nad and JBE viruses have obtained better CPE. However, they record inconsistencies of plaque formation and inhibition of CPE. They report loss of mouse pathogenicity following serial passage of JBE virus. Kissling(6) directed efforts primarily towards isolation of virus from tissues of infected animals and arthropods utilizing dog, guinea pig, hamster kidney cell and chick embryo tissue cultures. His results firmly establish the usefulness of hamster kidney cell tissue cultures for detection of EEE, WEE, Venezuelan, SLE, JBE, Murray Valley and Ilheus encephalitis viruses. Our purpose is to report routine preparation of monolayer roller tube tissue cultures from trypsinized hamster kidney by methods essentially identical to those employed for processing of monkey kidney tissues and to extend Kissling's findings regarding usefulness of these cultures as self-sufficient bio-assay system for certain members of arthropod-borne viruses. Evidence is presented that JBE agent has been serially propagated in this cell system and identified after passage by usual serological neutralization test. This demonstrates that *in vitro* method for study of arthropod-borne agents in the laboratory is feasible.

Materials and methods. Preparation of tissue cultures. In the beginning of our studies monolayer tissue cultures from hamster kidneys were prepared by the trypsinization procedures recently summarized by Melnick(7), with the following exceptions: (1) growth medium contained antibiotics to a final concentration of 400 units of penicillin, 2 mg of streptomycin, 20 units of polymyxin, and 25 units of mycostatin/ml; (2) 4% calf serum in 0.5% lactalbumin hydrolysate in Hanks balanced salt solution was used routinely for outgrowth and maintenance of cultures; (3) one pair of kidneys was processed at a time, age or sex was not considered in selection of animal; (4) no attempt was made to remove the pelvis of the hamster kidney; (5) one-half hour of pre-incubation at 37°C in approximately 50 ml of trypsin was employed, and stirring was carried out in approximately 50 ml. Usually 5 successive mixings and decantings at 7-minute intervals were sufficient to provide the desired volume of packed cells for the inoculum. The average yield was approximately .44 ml packed cells/pair of kidneys, which quantity was sufficient to seed 300 ml of medium. When dispensed to tubes in 1 ml amounts, the final number of cells/tube was approximately 150,000. More recently we employed trypsinization of a single pair of kidneys in 100 ml of trypsin for 3 to 4 hours at room temperature with equal success. Outgrowth of cells into a monolayer was generally complete in from 4 to 5 days, then tubes were shaken vigorously, the spent fluid decanted, and new medium with pH of 7.8 to 8.0 added. After overnight incubation the tubes were inoculated. Then tubes were maintained in a stationary slanted position at 37°. Monolayer cultures of hamster cells in glass bottles have also been prepared. However, demonstration of viral plaques has not been successful to date. *Virus preparations.* Aliquots of frozen virus suspensions prepared according to procedures outlined by one of us(8) served as the inoculum for our observations. Mouse and tissue culture passage levels of these viruses are indicated in the charts and tables of the *Results* section. In tests with JBE virus the Nakayama strain was employed. *Diluent.* Ten % normal calf serum (free from JBE antibody) in 0.5% lactalbumin hydrolysate medium served as diluent for titration of virus. *Harvest and storage* of infected tissue culture fluids was accomplished by addition of equal volume of normal calf serum to each tissue culture tube, shell freezing and then thawing at 37°C. The 50% fluids so obtained were pooled, dispensed to ampules in 0.5 ml to 5.0 ml, shell frozen and stored at −70°C. This procedure was deemed necessary to afford adequate protein protection for the labile JBE virus during freezing, thawing and storage. *Assays for viral activity* were accomplished by titrations in either tissue culture tubes or intracerebrally in mice.

TABLE I. CPE* of Selected Viruses on Hamster Kidney Tissue Culture, Dose 0.1 ml.

Viral agent	Group†	Mouse passage	Mouse titer‡	Range of dilutions tested§	End point CPE	Days observed‖
			Marked			
JBE	B	46	7.0	1–5	>5.0	3–5
WN	B	5	7.2	"	4.5	3–4
Uganda S	B	22	6.0	"	4.2	3–6
Ilheus	B	28	7.5	"	>5.0	3
Bunyamwera	?	39	6.6	"	"	3
Semliki Forest	A	8	7.2	"	"	3
			Moderate			
SLE	B	?	6.6	1–5	>5.0	3–5
Dengue I	B	121	?	1–3	1.5	5
" II	B	22	?	1–4	3.5	5
" Trinidad	B	54	?	1–3	2.5	5
			Negative			
Ntaya	B	17	5.9	1–5	<1	7
Zika	B	149	6.8	"	"	7
Bwamba	B	39	4.7	"	"	7
Polio I				1	"	7
" II				1	"	7
" III				1	"	7

* Cytopathogenic effect. † Casals' and Brown's serologic grouping. ‡ Reciprocal of log of mouse LD_{50}. § Reciprocal of log dilution. ‖ Range of days on which CPE became definite from the lowest virus dilution employed to the highest dilution showing CPE; or, if negative, the period of days cultures were observed.

For tissue culture tubes the unit volume of inoculum was 0.2 ml of 50% tissue culture fluid for routine passages or 0.1 ml of appropriate dilutions for titrations; for mouse inoculations the unit volume of inoculum was 0.03 ml. Neutralization tests for JBE virus were performed in usual manner for virus dilution tests and incubation was carried out for 2 hours at 37°C. *Screen tests for cytopathogenic effect* of certain arthropod-borne viruses were performed by thawing the stock mouse brain virus suspension at 37°C, diluting in 10% calf serum diluent and inoculating 0.1 ml of the indicated dilutions into each of 3 to 5 tubes of hamster kidney cell (HKC) tissue cultures. Daily microscopic observations were then made and results recorded as marked, partial or negative, according to whether or not all cells in the culture were markedly altered in appearance, only part of the cells were altered morphologically, or there was no observable difference between inoculated and control tubes maintained under identical conditions. No attempt was made to change the fluids in the cultures. If pH dropped excessively, it was adjusted by addition of a drop of sterile 1.4% $NaHCO_3$ solution.

Results. Susceptibility of hamster kidney tissue cultures to cytopathogenic effects of a variety of viruses. Table I records the findings noted when screen tests for cytopathogenic effects (CPE) of 13 arthropod-borne viruses and 3 polioviruses were performed. The screen tests were done as described above, and in Table I is recorded the range of dilutions used and the endpoint of the cytopathogenic effect. Moreover, the range of days on which CPE first was definite is indicated and also the longest period of observation when no CPE was noted.

It is apparent that arthropod-borne viruses tested fell into 3 groups as regards capacity to produce CPE within observation period of approximately one week. Moreover, this division into a group of those 6 agents which displayed marked CPE within 3 to 6 days, those 4 viruses which revealed only moderate CPE after 4 to 7 days, and those 3 agents which were without effect after 7 days' stationary incubation was not readily related to serological classification of these viruses according to serological groupings of Casals and Brown(9). Further work was limited to observations with Japanese B encephalitis virus as a representative member of Casals' Group

B for which no suitable tissue culture methods were available for routine, practical application.

Cytopathogenic effect of JBE virus. Fig. 1 illustrates the sequential appearance of hamster tissue following inoculation with JBE virus. The captions are self-explanatory and the photomicrographs at 24 and 72 hours post-inoculation reveal that marked morphological changes can frequently be noted as early as 24 hours following inoculation. In contrast to findings reported for the Detroit-6 cell line(3) no evidence of regeneration of tissue in cultures maintained for 21 days follow-

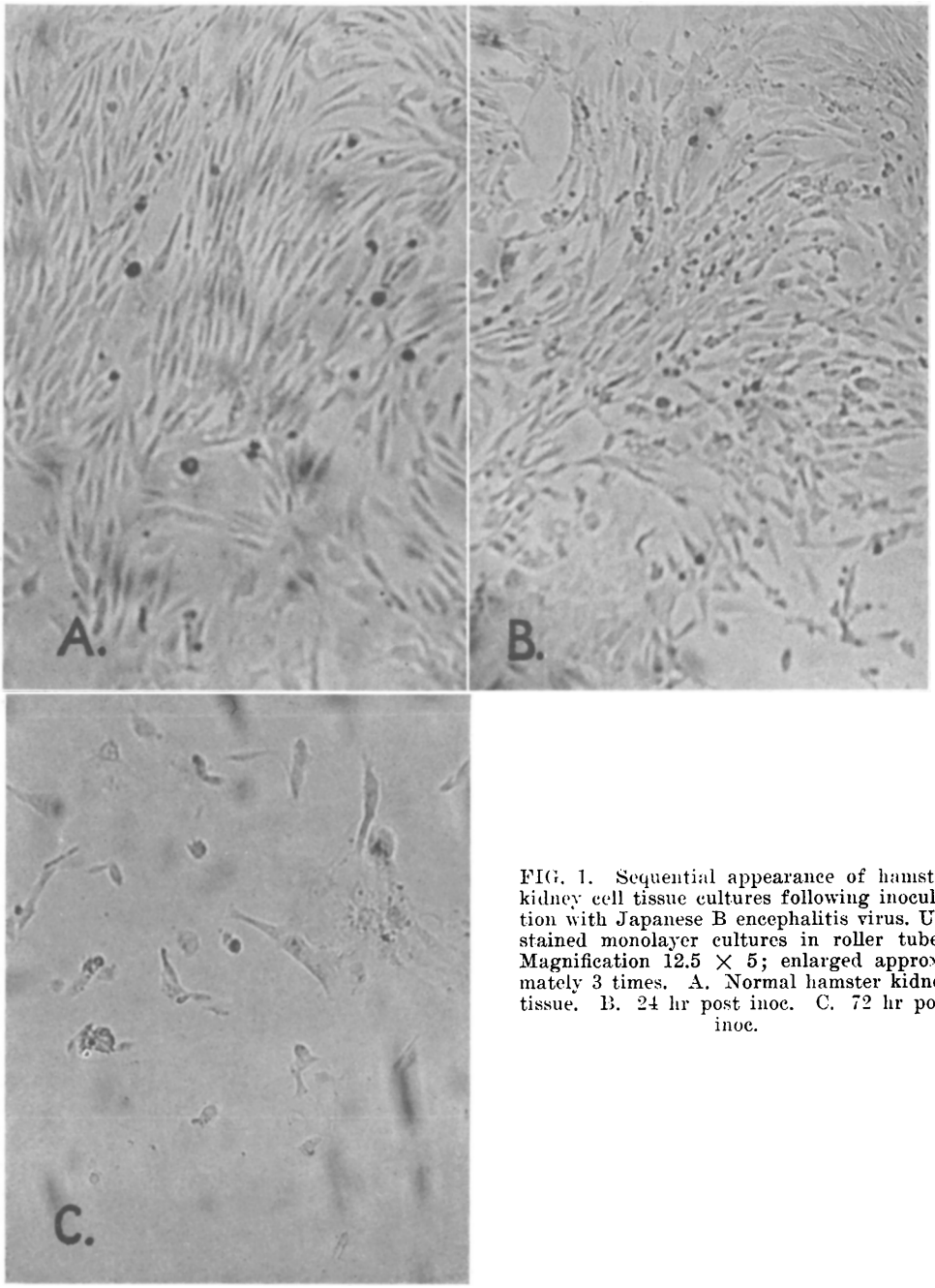

FIG. 1. Sequential appearance of hamster kidney cell tissue cultures following inoculation with Japanese B encephalitis virus. Unstained monolayer cultures in roller tubes. Magnification 12.5 × 5; enlarged approximately 3 times. A. Normal hamster kidney tissue. B. 24 hr post inoc. C. 72 hr post inoc.

TABLE II. Serial Passage of JBE Virus in Hamster Kidney Cell Tissue Cultures.*

Passage level	Dilution of mouse brain (neg log)	Mouse doses in inoculum† (6.9 ×)	Titer of yield‡ doses§/ml (× 1,000,000) Mice	TC	Day of harvest
P-47	3				
TC 1	4	100,000	7.	2	3
2	5	10,000	24.	5	2
3	6	1,000	189.	50	2
4	7	100	93.	10	2
5	8	10	1.9	2	2
6	9	1	1.9	20	2
7	10	.1	6.	20	2
8	11	.01	1.2	10	2

* Japanese B encephalitis virus in 47th mouse passage with LD_{50} titer of $10^{-8.37}$ used for inoculum; 0.1 ml of 1:1,000 dilution used to initiate cultures with subsequent transfers consisting of 0.2 ml of 50% tissue culture fluid in normal calf serum.

† Providing that no loss occurred.

‡ Tissue culture fluid titrated in 0.03 ml volumes in mice and 0.1 ml volumes in tissue cultures.

§ CPE_{50} for tissue cultures; LD_{50} for mice.

ing inoculation has been noted. It has been noted that with certain tissue culture yields the CPE has been delayed or markedly reduced in intensity when 50% tissue culture fluids or 1:10 dilutions of these fluids are inoculated into HKC. This finding, which suggests an interference effect, has not yet been adequately explained. In general, however, with virus dosages greater than 200 TCD_{50} CPE is complete within 72 hours. With smaller doses, 5 to 7 days are required for maximum effect. It should be recorded also that the JBE CPE has been an all-or-none phenomenon in dilutions of virus greater than 1:100. Thus, if even a few cells show evidence of morphological changes, the entire tube is eventually destroyed.

Serial propagation of JBE virus. The initial attempt at serial passage failed after 3 transfers, presumably because high concentrations of serum were not supplied to the tissue culture fluids at time of harvest prior to storage and subsequent passage. The second series of passages was accomplished by adding normal calf serum prior to storage at −70°C. Passages were intentionally not made on day of harvest, since any practical laboratory procedures must not depend on maintaining continuous passage of the test virus.

The results of serial propagation of JBE virus through 8 passages are summarized in Table II. Here serial passage in HKC resulted in 10^{-11} dilution of the original mouse brain, which is well beyond the extinction point of original infectivity. Moreover, tissue culture fluids harvested maintained a fairly constant titer of infectivity for the HKC of approximately 10^{-6}. Therefore, the original mouse brain suspension after a 10^{-11} dilution still contained essentially as many or more infective particles as it did originally, thus indicating that multiplication had occurred. In contrast to HKC titers, mouse infectivity titers have been more irregular and may be indicative that the virus is undergoing an adaptation to tissue culture.

Identification of passage virus by neutralization tests. After 6 serial passages in tissue culture the virus was identified by virus dilution neutralization procedure using as indicator of viral activity both the intracerebral inoculation of mice and the cytopathogenic effect of the virus on hamster kidney tissue cultures. Virus dilutions of 10^{-2} through 10^{-6} were employed with the immune serum using 4 cultures or 6 mice for each dilution. Titrations with normal control serum were carried out in dilutions of 10^{-3} through 10^{-7}. Essentially complete protection was afforded in all dilutions by the immune serum, giving a virus titer of less than 10^{-2} while virus titration in the presence of normal serum gave $10^{-5.5}$ and $10^{-5.4}$ respectively in tissue cultures and in mice. Thus, the results of the 2 tests were essentially identical.

Discussion. We emphasize that information in this report is little more than a progress report and that much additional work must be done before it can be said that there now exists a usable and practical tissue culture method for laboratory study of additional members of the arthropod-borne viruses. However, it is interesting to speculate upon certain important results which could be foreseen if a truly practical "test tube method" were available for routine use. First, it would mean that many of our smaller public health laboratories would have at hand a convenient method for supporting the physician's clinical diagnosis of arthropod-borne viral en-

cephalitis infections by attempted direct isolations of the virus. Secondly, it should be possible to gain fundamental knowledge about the mode of spread of the encephalitis viruses within a monolayer of cells or through the fluid medium such as we now have for poliovirus and others of the more stable viruses. Thirdly, such a system may well provide a source of relatively pure virus particles for preparation of diagnostic antigens or inactivated vaccines. Lastly, continued passage of virus in tissue culture may lead to selection of attenuated strains of virus suitable for production of live virus vaccines such as are now available for prophylaxis against yellow fever.

Summary. (1) Monolayer HKC tissue cultures can be prepared on a routine basis by methods essentially identical with those currently in use for preparation of monkey kidney tissue cultures. In screen tests with 13 arthropod-borne agents and 3 types of poliovirus, HKC proved markedly susceptible to the CPE of Japanese B encephalitis, West Nile, Uganda S, Ilheus, Bunyamwera, and Semliki Forest viruses, only moderately susceptible to effects of the St. Louis encephalitis agent and the New Guinea C, Hawaiian, and Trinidad strains of dengue virus. In contrast, the Ntaya, Zika and Bwamba agents and the 3 types of poliovirus failed to show CPE. (2) Evidence has been presented for serial propagation of JBE virus through 8 serial transfers in HKC tissue cultures. CPE was eventually complete. After 6 passages the virus yield was greater than the starting inoculum, although the dilution factors had carried beyond the extinction point of infectivity of the original inoculum. (3) Identity of the Japanese B encephalitis virus after 6 serial passages in tissue culture was established by neutralization tests performed both in tissue cultures and in mice and thus indicates that the "test tube" procedure may prove useful as a serological tool in the laboratory.

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Reactivity of Globulins from Rheumatoid Sera in the Latex-Fixation Test.* (23828)

MELVIN RHEINS, FRANCIS W. MCCOY, AND ROBERT L. WALL
(Introduced by Matt C. Dodd)
Departments of Bacteriology and Medicine, Ohio State University, Columbus

While discussing the various parameters of the latex-fixation test for rheumatoid arthritis, Singer and Plotz(1) reported that 11% of 150 rheumatoid sera agglutinated a standardized suspension of latex particles from which globulin had been deliberately omitted. These sera were those which were strongly positive with the standard "antigen", *i.e.* with a latex particle suspension containing 25 γ of human γ-globulin/ml. The tentative explanation presented was that, in such instances, the latex particles became coated with γ-globulin, or a fraction thereof, from the pa-

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