laboratories where arbovirus serological tests are performed. Minor modifications should make it applicable for a variety of viruses in group B, at least.

Summary. Hemagglutinins (HA) of JBE virus prepared from infected HKC cultures were masked by serum, an essential ingredient of good nutrient medium. By removing the serum containing medium at the first sign of CPE and substituting a serum-free medium 199 at pH 8.0, until harvesting 16 hours later, a high titered HA was obtained. Reducing the amount of culture medium resulted in a further proportional increase in hemagglutinins and an HA titer of at least 1:1024 was easily obtained. Factors such as adaptation of the virus strain to tissue culture, multiplicity of infection as respects the inoculum, and delayed harvest did not seem to play any important role in HA titer. However, the presence of a stabilizer (H.Al.) in the serum-free medium may have a minor effect on HA titer at pH 7.0. A direct relationship was usually found between the infectious and HA titers in a freshly harvested serum-free medium. Stability of hemagglutinin was demonstrated for at least 8 days at 30° and 37°C in medium 199 containing 2% H.Al. at pH 7.8 though the infectivity titer decreased rapidly. At 5°C the HA titer was preserved without detectable decrease for at least 18 months and such stability was not dependent on the pH of the harvest or the presence of any added stabilizer in the medium. Such HA was noninfectious. The simple technique employed to achieve high-titered hemagglutinin from tissue culture and its stability together with loss of infectivity at refrigerator temperature offer a valuable substitute for the suckling mouse brain antigen for this, and at least some other group B arboviruses and probably others.

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Japanese B Encephalitis Virus Vaccines from Tissue Culture* VII. Formalin Inactivated Nakayama Strain Vaccine. (31258)

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In previous articles(1-4) a formalin inactivated Japanese B encephalitis (JBE) vaccine was described which was prepared from an attenuated strain of JBE virus, derived from OCT-541, grown in hamster kidney cell (HKC) culture. When administered to human volunteers it was shown to elicit significant antibody formation, apparently superior to that produced by earlier crude vaccines from virus produced in mouse brain and chick embryo *in vivo*. The Nakayama strain was the virus used in these earlier vaccines(5,6)

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and was specified in the licensing requirements for the JBE vaccine made in the United States(7). For comparative purposes a study was carried out of the inactivation and antigenicity of the Nakayama strain grown in tissue culture(8).

Materials and methods. The Nakayama strain following 47 mouse passages was passed 33 times in HKC culture(9,10). The seed virus in HKC culture medium with an equal amount of inactivated normal calf serum (INCS) was stored at —70°C. The virus was harvested in the same manner as the attenuated strain in HKC culture with human albumin (H.Al.) and no serum, in medium 199(1) which replaced a serum containing medium 16 hours before harvest time. Incubation was at 37°C instead of 30°C. Some virus suspensions were filtered through a Millipore disc (pore size 0.22 μ) before inactivation, others remained unfiltered. Inactivation was with 1:4000 formalin as previously described for the attenuated(2), at either 30° or 37°C and at pH 7.1. All samples removed at intervals during inactivation of one lot were simultaneously titrated for infectivity in HKC cultures. Pre-inactivation preparations were titrated for hemagglutinins as were also, on occasion, samples removed during or after inactivation. The inactivation course of each vaccine lot was plotted graphically. Infectivity and HA titrations were made by conventional methods as described previously (1). Potency tests were performed by a slight modification of the mouse challenge test developed by Sabin *et al*(5) and described earlier(3) and are recorded as the minimal immunogenic dose (MID). Serum neutralizing antibody tests were also used for determining potency following vaccine inoculations in mice and monkeys(3), and are recorded as $\log_{10}$ neutralization indices (NI)(11).

Experimental results. Virus yield in fluid and complete harvests. Four pools of virus were prepared. The infectivity and HA titrations were 1:512/0.4 ml and $10^{-8.0}$/0.1 ml respectively for both the fluid and complete harvests for a typical pool at about pH 7.0.

Half-life of the Nakayama virus at 30° and 37°C and pH 7.1. The estimated half-life of the Nakayama strain under these experimental conditions was 11 and 4 hours at 30° and 37° respectively.

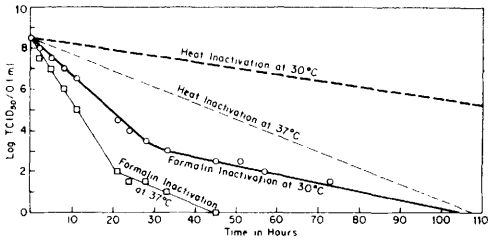

FIG. 1. Effect of heat and of 1:4000 formalin on JBE virus (Nakayama strain) in 199 medium containing 2% human albumin at 30° and 37°C, pH 7.1.

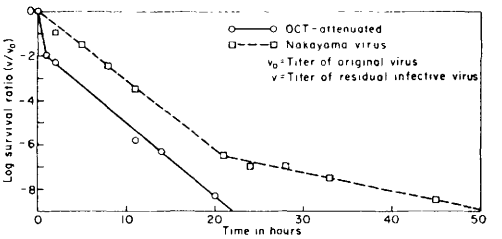

FIG. 2. Formalin inactivation (1:4000) of Nakayama and OCT-attenuated 24°C strains of JBE virus in 199 medium containing 2% human albumin at 37°C, pH 7.1.

Formalin inactivation. A fluid harvest, pH 7.1, was inactivated by 1:4000 formalin, one part at 30° and another at 37°C. The reaction was allowed to proceed in sealed ampoules submerged in water at the required temperature and samples were removed and frozen at intervals for titration. Fig. 1 presents inactivation curves at the 2 temperatures. The effect of heat inactivation alone obtained during the half-life determinations of the previous experiment is superimposed. The formalin activation curves were biphasic with the inflections occurring late in the reaction.

Potency testing. Two lots of virus suspension were prepared as those described above in medium 199 containing 2% H.Al., one a fluid harvest, the second a complete harvest (disrupted cells and fluid). Both were centrifuged and the supernatant fluid inactivated by 1:4000 formalin at pH 7.1 and 37°C. The infectivity and HA titers of both fluid and complete harvests were $10^{-8.0}$/0.1 ml, and 1:512 respectively. The formalin inactivation proceeded parallel with that shown

TABLE I. MID* and NI† at Various Stages of Inactivation for a Nakayama Strain Formalin Inactivated Vaccine Prepared from Fluid and Complete Harvests of Virus of Titer 10^{-8}/0.1 ml.

	Fluid harvest			Complete harvest	
Parameter tested	Single inact. period	Double inact. period	Triple inact. period	Double inact. period	Triple inact. period
MID* in ml	.02	.051	.054	.015	.038
Log NI† of pooled sera of mice receiving 1:20 vaccine	1.8	1.5	1.8	1.8	2.0
Log NI† of pooled sera of mice receiving 1:80 vaccine	2.0	1.5	1.6	1.2	1.5

* MID = minimal immunogenic dose. † NI = neutralization index.

in Fig. 1 and no live virus was detected after 40 hours in either harvest. Inactivation was continued for a total of 120 hours. The HA titer after 80 hr ("double" inactivation period) was 1:256.

The results of potency tests at the "double" and "triple" inactivation periods for both fluid and complete harvests, and in addition of the "single" inactivation period for the fluid harvest are shown in Table I. The challenge virus in the 5 mouse potency tests met all necessary criteria in that it had an intracerebral (i.c.) titer ranging from $10^{-9.0}$ to $10^{-9.5}$ and killed from 91 to 100% of the control unvaccinated mice.

In another experiment, a complete harvest of higher titer was inactivated with formalin for the "triple" period in the same manner, after filtration. The pre- and post-filtration infectivity titers were $10^{-8.5}$ and $10^{-8.3}$/0.1 ml respectively while the HA titer was 1:512 in both cases. When the inactivated preparation was tested for potency in mice, it gave a MID of 0.02 ml.

Monkey potency test. The above lot of vaccine (MID of 0.02 ml) was tested by the monkey potency test. Two monkeys (No. 14 and No. 15) were bled prior to vaccination, 26 days after the first dose of vaccine, and finally exsanguinated, one 80 days and the second 83 days after the first injection. Monkey No. 14 had a NI of 3.7 logs at 26 days and it increased to 5.0 logs at 80 days. Monkey No. 15 had a NI of 3.5 logs at 26 days and it remained the same at 83 days.

Discussion. From this study certain differences were noticed between the Nakayama strain of JBE virus and the previously reported OCT-attenuated strain(1-3). Table II summarizes some of the important differences between these two strains. It is obvious that the older, mouse adapted Nakayama strain even after prolonged adaptation to HKC culture and with high infectivity titers is a far less suitable strain for tissue culture vaccine (formalin inactivated) than the previously tested attenuated variant of OCT-541 virus. In fact, only the preparation with the highest preinactivation titer ($10^{-8.3}$/0.1 ml) met the minimal MID of 0.02 ml required for the previously licensed chick embryo vaccine(7).

This apparent lower potency of the final vaccine from the Nakayama strain in comparison with that of the OCT-attenuated strain is probably due in large part to the nature of inactivation of the Nakayama strain. The longer period required to achieve a single inactivation period for the Nakayama strain and the change in the slope of the reaction at a low survival ration may subject the major mass of virus inactivated early to overtreatment resulting in impairment of antigenicity. If the inactivation is to be extended to the triple period, as appears to be required currently in the United States for new virus vaccines, the antigenicity of the Nakayama will be more seriously affected as demonstrated here. A difference in antigenicity between the two strains of JBE virus prior to inactivation might also be responsible for the better potency of vaccines prepared from the attenuated OCT-541 strain, but evidence for such was not sought.

Summary. The Nakayama strain of JBE virus which, was grown in HKC and harvested

TABLE II. Comparison Between the Nakayama and OCT-Attenuated Strains of JBE Virus and Their Vaccines.

Parameter	OCT-attenuated virus	Nakayama virus
Infectivity of complete *vs* fluid harvest, pH 7.0	1.5 log higher	identical
Half-life at 30°C	14 hr	11 hr
" " " 37°C	5½ "	4 "
Formalin (1:4000) inactivation curve (Fig. 2)	biphasic	biphasic
Inflection	early at a survival ratio of 10^{-2} or more	late at a survival ratio of 10^{-5} or less
"Single" inactivation period with original titer of $10^{-8.5}$/0.1 ml		
at 30°C	45 hr	106 hr
at 37°	33 "	45 "
Early phase of inactivation	very rapid	slower and parallel to late phase of OCT-attenuated
Late phase of inactivation	much faster than heat	very slow and parallel to heat
Potency testing by mouse MID		
MID at "triple" period as related to preinactivation infectivity $TCID_{50}$	MID of .011 ml for $10^{-7.5}$/.1 ml; acceptable	MID of >.02 ml (.038 and .054) for $10^{-5.0}$/.1 ml. Not acceptable
Prolongation of inactivation from single to triple period	MID not affected	MID increased
MID for preinactivation titer of $10^{8.3}$ $TCID_{50}$/0.1 ml for triple period	.0089 ml; excellent	.02 ml; borderline
Potency testing by monkey potency test. Range of NI in relation to $10^{-8.3}$. Preinactivation titer	4.8–6.8 log NI	3.5–5.0 log NI

in medium 199 with 2% H.Al., was inactivated by 1:4000 formalin and tested for potency. The inactivation curves at 30° and 37°C were biphasic with the inflection points occurring late in the reaction at a low survival ratio of 10^{-5} or less. The second phase of inactivation proceeded at a rate comparable to that of heat of the same degree alone, resulting in a great prolongation of the time required for inactivation. The antigenic potency appeared to decrease with prolongation of the inactivation time and when the triple inactivation period was achieved the potency of most lots was unacceptable. The OCT-attenuated strain of JBE virus prepared previously in the same manner and with comparable infectivity titers prior to inactivation was inactivated in less than half the time required by the Nakayama virus and consistently yielded a more potent vaccine.

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