10. Schmitt, G. H., Fed. Proc., 1964, v23, 329. (Abst.)
11. Hulet, W. H., Baldwin, D. S., Biggs, A. W., Gombos, E. A., Chasis, H., J. Clin. Invest., 1960, v39, 389.
12. Smith, H. W., Principles of Renal Physiology, Oxford Univ. Press, New York, 1956, p208.
13. Vagnucci, A. I., Lauler, D. P., Hickler, R. B., Thorn, G. W., Circulation, 1964, v29, 523.
14. Goldsmith, C., Rector, F. C., Jr., Seldin, D. W., J. Clin. Invest., 1962, v41, 850.
15. Kamm, D. E., Levinsky, N. G., ibid., 1965, v44, 1144.
16. Ek, J., Scand. J. of Clin. & Lab. Invest., (Suppl. 19), 1955, v7, 1.
17. Levinsky, N. G., Lalone, R. C., J. Clin. Invest., 1963, v42, 1261.
18. Rector, F. C., Jr., Van Giesen, G., Kiil, F., Seldin, D. W., ibid., 1964, v43, 341.
19. Sonnenblick, E. H., Cannon, P. J., Laragh, J. H., ibid., 1961, v40, 903.
20. Dahl, L. K., Smilay, M. G., Silver, L., Spraragen, S. C., Nature, 1961, v192, 267.

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A High Titered Hemagglutinin in Tissue Culture Prepared from Japanese B Encephalitis Virus.* (31257)

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Since tissue culture (TC) methods were introduced in arbovirus work, may have attempted to utilize infected fluid as a source of hemagglutinin (HA) antigen as a substitute for tissues and the tedious processing these require in the *in vivo* method currently employed(1). Non-specific inhibitors in certain sera used in TC work were found to mask HA activity, and techniques such as acetone extraction, protamine sulphate or genetron treatment were described for recovering active hemagglutinin(2-4). Other workers used inhibitor-free media or subsequent concentration to achieve higher hemagglutinin titers(5,6); still the HA titers seldom exceeded 1:64.

In search for the optimum method to obtain a Japanese B encephalitis (JBE) virus of high titer from hamster kidney cell (HKC) culture for vaccine purposes, hemagglutinins were explored(7). In many trials with a variety of media including those with serum albumin, it had been found that a minimum of 2% whole serum (usually calf) appeared to be essential to maintain the cells adequately during the early growth phase of the virus to obtain maximal virus titers(8). However, it was found possible when cytopathic effect (CPE) was first noted to remove this serum containing medium, wash the cells, replace with many varieties of serum-free media, harvest 12 to 16 hours later and still recover equally high infectivity titers(7,8). This also provided a direct, easy method for producing high titered, stable hemagglutinin from infected HKC. The relationship of hemagglutinin to infectivity titers was studied.

Materials and methods. Virus strain used: An attenuated strain of JBE virus, designated as OCT-541, line 35-24, plaque 4-5, was used. This is a strain adapted to grow at relatively low temperatures in HKC through many serial passages(9,10).

Preparation of virus suspension: The virus was grown in HKC monolayers in 3 oz bottles at 30°C in the presence of maintenance medium previously described(7), but containing in part 4% normal calf serum (NCS) and lactalbumin hydrolysate. When CPE started to appear the fluid was removed, cell sheets were washed thoroughly with Hanks' balanced salt solution (BSS) and bottles replenished with 7.0 ml of one of a variety of media.

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TABLE I. Infectivity and HA Titers in Maintenance Medium Containing 4% Normal Calf Serum and in Subsequently Added Medium 199 Containing 2% Human Albumin.

Medium	HA titer/0.4 ml*	Infectivity titer/0.1 ml†
Maintenance	<2	$10^{-6.0}$
Medium 199	64	$10^{-6.5}$

* Reciprocal of highest dilution showing complete hemagglutination.

† Log_{10} of $TCID_{50}$ as calculated by Reed-Muench method.

After 16 hours of further incubation the virus was harvested(7).

Virus assay. Hemagglutinin titrations were performed in lucite plates in a modification of the method described by Clarke and Casals (1). Serial 2-fold dilutions of virus suspension were mixed with equal amounts of 0.4% goose erythrocytes at pH 6.4 at room temperature for 1 hr. Infectivity titers were determined by inoculating 0.1 ml of each of 10-fold dilutions in each of 4 HKC monolayer tubes which were subsequently incubated at 37°C until read for CPE.

Experimental results. Effect of medium composition of hemagglutinin: The virus was grown in the presence of maintenance medium containing NCS which was replaced on appearance of CPE by Parker's 199 medium containing 2% human albumin (H.Al.). Infectivity and HA titrations were performed on both the early and the late virus harvests, each in a different medium. The results are shown in Table I. The HA titer which was not detectable in maintenance medium increased more than 32-fold in the 199 medium with 2% H.Al. while the difference in infectivity appeared to be only 3-fold.

Effect of calf serum and phenol red on hemagglutinin. It had been reported that certain normal sera contain non-specific hemagglutinin-inhibitors to some arboviruses(2-4). Likar *et al*(6) found that by omitting phenol red from the maintenance medium, titers of hemagglutinins could be increased 2- to 4-fold with central European tick-borne encephalitis virus.

Table II shows the results obtained with harvests again made 16 hours after a medium change, using media with and without phenol red and/or calf serum. It is clear that the hemagglutinin inhibition was due to calf serum. Phenol red did not seem to have any inhibitory effect on HA within the experimental conditions. The slightly higher infectivity titer with calf serum might reflect the stabilizing effect of NCS on infectivity of released virus.

Effect of certain factors on hemagglutinin in serum-free medium. (a) *Human albumin.* Medium 199 with different H.Al. concentrations at pH 7.0 was used as the final harvest medium. The results are shown in Table III. The two higher concentrations (4% and 8%) appear to offer some small HA titer increase with an associated infectivity increase. (b) *pH of the medium.* Medium 199 of pH values from 6.8 to 8.0, containing no serum or H.Al. was next used as the final medium. It can be seen (Table IV) that by increasing the pH of the harvest medium the infectivity and HA titers were progressively increased up to a pH of 7.9, at least. (c) *Amount of medium.* In the previous experiments, 7 ml of 199 medium

TABLE II. Effect of Calf Serum and of Phenol Red (P.R.) on HA Titers.*

With calf serum		Without calf serum	
With P.R.	Without P.R.	With P.R.	Without P.R.
<2	<2	64	64

* All infectivity titers ranged between $10^{7.3}$ and $10^{7.7}$, the highest titer occurring each time with calf serum.

TABLE III. Effect of Concentration of Human Albumin on HA and Infectivity Titers in Medium 199.

Medium	HA titer/0.4 ml	Infectivity titer/0.1 ml
With 2% H.Al.*	64	$10^{7.0}$
" 4% "	128	$10^{7.25}$
" 8% "	128	$10^{7.5}$

* H.Al. = human albumin.

TABLE IV. Effect of pH on Infectivity and HA Titers in 199 Medium Alone.

pH of medium	HA titer/0.4 ml	Infectivity titer/0.1 ml
6.80	<2	$10^{5.0}$
7.15	32	$10^{6.0}$
7.32	64	$10^{6.7}$
7.60	256	$10^{7.2}$
7.90	512	$10^{8.0}$
8.00	512	$10^{8.2}$

TABLE V. Relationship Between Amount of Medium and Titers of HA and Infectivity at pH 8.0.

ml of medium	HA titer/0.4 ml	Infectivity titer/0.1 ml
2.5	1024	$10^{8.3}$
5.0	512	$10^{8.0}$
10.0	256	$10^{7.8}$

TABLE VI. Effect of Time of Harvest on HA and Infectivity Titers in Medium 199 with 2% Human Albumin at pH 8.0.

Hr after adding medium	Degree of CPE	HA titer/0.4 ml	Infectivity titer/0.1 ml
16	+1	256	$10^{8.3}$
40	+3	256	$10^{7.7}$
64	+4	256	$10^{7.5}$

were used for harvesting the virus from HKC monolayers grown in 3 oz prescription bottles. The following experiment (Table V) showed that decreasing the quantity of final harvest medium for a constant number of cells and inoculum resulted in inversely proportionate increases of the HA and infectivity titers.

Effect of delayed harvest on hemagglutinin titer. Salminen(11) reported that with certain arboviruses including JBE, a late harvest (very advanced or complete CPE) resulted in a higher hemagglutinin titer, and suggested that the HA titer reflected the cumulated dead and live virus in the tissue culture fluid. In the following experiment medium 199 containing 2% H.Al. at pH 8.0 was added as usual at the time of early CPE and harvested at different intervals thereafter as shown in Table VI. The results showed that under these conditions HA titers were not detectably increased or decreased by delaying the harvest, whereas infectivity titers were reduced.

Effect of virus multiplicity of inoculum on HA production. One report indicates that the HA titer is independent of the inoculum titer (3). In the following experiment different virus multiplicities were used for infecting tissue monolayers in maintenance medium. Medium 199 containing 2% H.Al., pH 7.0 was added as usual after the appearance of CPE and harvested 16 hours later. The results (Table VII) indicate that under these circumstances multiplicities ranging from 1:0.25 to 1:1500 had no advantageous effect on HA titer and no significant effect on infectivity.

Hemagglutinin of OCT-wild vs *OCT-attenuated strain.* Since the OCT-attenuated strain used represented a series of more than 50 passages in HKC(9,10), it was of interest to compare it with the OCT strain at a very early passage level, passage 4, (OCT-wild) to determine whether this adaptation might have any influence on hemagglutinin and/or infectivity titers under identical conditions. Table VIII shows the results of an experiment in which the viruses were prepared in medium 199 containing 2% H.Al. at pH 8.0. Identical infectivity titers were obtained but the earlier passage level showed a 2-fold higher HA titer, one of the highest obtained (1:1024), yet not necessarily significantly different from the other.

Effect of certain temperatures on hemagglutinin stability. (a) *Stability at 30° and 37°C.* A virus harvest in medium 199 containing 2% H.Al. at pH 7.8 was inactivated at both 30° and 37°C. Inactivation was carried out in ampoules immersed in water at either temperature, shell-frozen when removed, stored at −70°C until the experiment was terminated and finally titered for infectivity and hemagglutinin. The virus maintained a constant HA titer for at least 200 hours at both temperatures whereas infectivity decreased at a half-life of 18 and 6 hr at 30°

TABLE VII. Effect of Virus Multiplicity on HA and Infectivity.

Virus multiplicity*	Time in days for CPE appearance	HA titer/0.4 ml	Infectivity titer/0.1 ml
1:1500	5	64	$10^{6.8}$
1:150	4	64	$10^{6.5}$
1:15	3	64	$10^{6.3}$
1:0.25	3	64	$10^{6.5}$

*$TCID_{50}$ per number of hamster kidney cells.

TABLE VIII. Hemagglutinin and Infectivity Titers of the OCT-Wild and -Attenuated Strain of JBE Virus.

Virus	HA titer/0.4 ml	Infectivity titer/0.1 ml
OCT-wild	1024	$10^{8.7}$
OCT-attenuated	512	$10^{8.7}$

TABLE IX. Stability of Hemagglutinin in Different Media at 4-6°C After Storage for 18 to 19 Months.

Medium	Immediate titers: Infectivity/0.1 ml	Immediate titers: HA 0.4 ml	†HA titers after storage
Medium 199 alone, pH 8.0	$10^{7.3}$	512	512
Medium 199 with 2% H.Al., pH 8.0	$10^{8.5}$	256	512
Medium 199 with 2% LAH,* pH 8.0	$10^{7.8}$	256	256
Medium 199 with 2% H.Al., pH 7.0	$10^{6.5}$	64	64
Medium 199 with 2% LAH, pH 7.0	$10^{7.3}$	256	256

* LAH = lactalbumin hydrolysate.
† Infectivity titers were $<10^{6}$.

and 37° respectively. (b) *Stability at refrigerator temperature.* The infectivity and HA titer of virus suspensions in different media were tested immediately after harvest and after storage in the refrigerator (4-6°C) for 18 to 19 months. As seen in Table IX, hemagglutinin was stable for that period of storage, whereas infectivity was destroyed.

Discussion. It is relatively easy to produce large volumes of infected tissue culture fluid for many arboviruses, and if hemagglutinins are present in relatively high titer this will provide a valuable supplement to the more commonly used antigens prepared from suckling mice. However, certain non-specific inhibitors, probably lipid in nature, present in many TC nutrient media can mask the HA activity(2-4). Certain normal sera, including calf, were reported to exert such inhibitory effect on JBE hemagglutinin. Several investigators have tried to overcome this masking effect. Diercks *et al*(3) succeeded in demonstrating hemagglutinins in infected HKC cultures by using 4% serum from a selected horse, known to have a minimal amount of normal inhibitors. Acetone extraction was reported to recover arbovirus hemagglutinin, including JBE, from inhibitor containing TC fluids(12). Acetone extraction produces workable HA antigens but requires use of large amounts of acetone, is laborious and associated with a danger of laboratory infection. Likar *et al*(6) found that concentration by ultra-filtration of infected fluid prior to acetone extraction will increase the hemagglutinin titer. Protamine sulphate and genetron have also been described as methods for removal of hemagglutinin inhibitors(5). Salminen, using an inhibitor-free medium containing bovine plasma albumin instead of serum was able to demonstrate hemagglutinin of arboviruses (including JBE) from a continuous line of human amnion cells(5). The hemagglutinin titer in the latter case for JBE virus was 1:64 and it rarely exceeded that in the other studies mentioned.

Experience with HKC culture showed that the monolayers were adversely affected when serum-free medium was used from the time of inoculation, resulting in lower virus yield, and that 2% H.Al. could not replace serum during the period of early virus growth(8). Consequently, virus was inoculated in the presence of calf serum, but at the appearance of early CPE, serum-free medium 199 with or without H.Al. was added to be harvested about 16 hours later(7). The pH of the added medium 199 was an important factor in determining the HA titer of the virus harvest. Increasing the pH of the medium from 6.8 to 7.9 resulted in more than a 256-fold increase in hemagglutinin titer (Table IV). At pH 8.0 high titered hemagglutinin was obtained irrespective of the presence of H.Al. or any stabilizer in the medium (Table IX). Further increase in HA titer was achieved by reduction of the amount of the final medium used (Table V). Such a concentration procedure is simpler than vacuum dialysis using an ultrafilter or any procedure used after harvesting, all requiring an additional handling of infectious fluids. The stability of the hemagglutinin in medium 199 was remarkable. HA titers were maintained at 4°C for more than 18 months irrespective of the pH of the harvest and the presence of stabilizer in the medium (Table IX). This offers another advantage over certain of the mouse brain antigens. Since it has been shown by others(2) that antigens prepared from HKC cultures and those from suckling mouse brain did not show any essential difference in reactions with homologous or cross reacting sera, this *in vitro* method can be recommended for use in research, diagnostic and public health

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.

1. Clarke, D. H., Casals, J., Am. J. Trop. Med. & Hyg., 1958, v7, 561.
2. Kundin, W. D., Diercks, F. H., Virology, 1960, v10, 153.
3. Diercks, F. H., Kundin, W. D., Porter, T. J., Am. J. Hyg., 1961, v73, 164.
4. Salminen, A., Virology, 1962, v16, 201.
5. ——— Ann. Med. Exp. Fenn., 1962, v40, 174.
6. Likar, M., Buckley, S. M., Clarke, D. H., Virology, 1962, v18, 648.
7. Darwish, M. A., Hammon, W. McD., J. Immunol., 1966, v96, 691.
8. Hammon, W. McD., Rohitayodhin, S., Rhim, J. S., ibid., 1963, v91, 295.
9. Rohitayodhin, S., Hammon, W. McD., ibid., 1962, v89, 589.
10. ——— ibid., 1962, v89, 823.
11. Salminen, A., Acta Path. et Microbiol. Scand., Suppl., 1962, v154, 343.
12. Buckley, S. M., Srihongse, S., Proc. Soc. Exp. Biol. and Med., 1963, v113, 284.

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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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