ARBOVIRUS INFECTIONS IN REPTILES: STUDIES ON THE PRESENCE OF JAPANESE ENCEPHALITIS VIRUS ANTIBODY IN THE PLASMA OF THE TURTLE, *TRIONYX SINENSIS*

K.F. Shortridge*, A. Oya**, M. Kobayashi** and D.Y. Yip***

*Department of Microbiology, University of Hong Kong, Pathology Building, Queen Mary Hospital Compound, Hong Kong, **Department of Virology and Rickettsiology, National Institute of Health, Shinagawa-ku, Tokyo 141, Japan, and ***Department of Zoology, University of Hong Kong, Hong Kong.

INTRODUCTION

Japanese encephalitis (JE) virus is an important human and animal pathogen in the Oriental Region. In Japan and Taiwan, water-birds such as egrets and herons are known to be infected by JE virus but non-immune swine are probably the most important hosts for infected vector mosquitoes before the onset of JE virus epidemics in man (Buescher and Scherer, 1959; Hurlbut, 1964; Konno *et al.*, 1966; Detels *et al.*, 1970).

Accumulating evidence indicates that reptiles, too, are associated with arboviruses (see review by Hoff and Trainer, 1973). Some may be overwintering hosts as appears to be the case with snakes of the genus *Thamnopsis* for western equine encephalitis (WEE) virus (Thomas and Eklund, 1960 and 1962). Negative information was obtained about JE virus from this aspect (Mifuna *et al.*, 1969); Japanese workers have experimentally infected the lizard *Takydromus tachydromoides* (Doi *et al.*, 1968) while combined virological/serological studies on two local Korean snakes *Elaphe rufodorsata* and *E. schrenkii* suggest they may be hosts for this virus (Lee, 1968; Lee *et al.*, 1972). Recent studies in Hong Kong have shown that 71% of cobra *(Naja naja)* snakes originating from neighbouring Kwangtung Province were antibody positive for JE virus in hemagglutination inhibition and virus neutralization tests and that there were seasonal differences in immu-

noglobulin class and distribution (Shortridge *et al.*, 1974) suggesting a similar relationship to the one reported for *Thamnopsis* and WEE virus (Thomas and Eklund, 1960 and 1962). Additionally, current serological studies to be reported from the authors' laboratories indicate that the banded krait *(Bungarus fasciatus)* and the rat snake *(Ptyas korros)* also have a high incidence of infection by JE virus.

These findings prompted us to study the turtle with a view to extending further the Orders of reptiles susceptible to JE virus infection. This animal was particularly attractive as it has been reported to be susceptible to arbovirus infection in other geographical areas (Hoff and Trainer, 1973). A soft-shelled species of fresh-water turtle *Trionyx sinensis* Wiegman *(Amyda sinensis* W.) was chosen as it occurs in neighbouring Provinces where *N. naja* is also found (Pope, 1935). The findings and possible significance of JE virus infection in *T. sinensis* detected by recognized serological procedures is the subject of this communication.

MATERIALS AND METHODS

Turtle plasma

Male soft-shelled turtles (*Trionyx sinensis* Wiegman) were caught at Wuchow, Kwangsi Province near the border with Kwangtung Province, China and shipped within a week to dealers in Hong Kong. Newly arrived

turtles estimated to be 3-4 years old were purchased and kept in fresh water tanks for 24 hours prior to blood collection. The head was severed with a large, sharp knife without anaesthesia and the blood issuing from the carotid artery collected into clean, heparinized centrifuge tubes which were then spun at high speed in a bench centrifuge for 15 minutes. The clear plasma was decanted into plastic vials and stored at –15°C until required.

The turtles were collected on a monthly basis apart from March-May when specimens were unavailable. As in a previous study on the cobra (Shortridge et al., 1974), the turtles were placed into one of two groups based on the season in which they were collected:- (i) autumn/winter, October to March, 43 turtles and (ii) spring/summer, April to September, 32 turtles.

Other reptiles

To gain some information on the behaviour of reptile non-antibody inhibitory substances in the tests described below, the sea snake, *Hydrophus cyanocynctus*, which is widely distributed throughout the Oriental continental shelf region, was included in this study on the grounds that it could not have been bitten by mosquitoes and would therefore be free of specific arbovirus antibody. Plasma was obtained from six of these sea snakes which were bled as previously described (Shortridge et al., 1974). Sea turtles were unavailable.

Hemagglutination inhibition (HAI) tests

Antibody was quantitated with the Nakayama strain of JE virus in the microhemagglutination test using procedures described by Clarke and Casals (1959). Prior to testing for HAI activity, samples were titrated for their gander cell agglutinin content, then treated with kaolin and packed cells (as required) to remove non-specific inhibitor (NSI) and natural agglutinins, respectively,

thereby resulting in a ten-fold dilution of the sample. Where indicated, trypsinized human group "O" erythrocytes were used, with equal sensitivity in the HAI test, as an alternative to gander cells to eliminate the agglutinin adsorption step (Shortridge and Hu, 1974).

Virus infectivity neutralization (VN) tests

Antibody was also detected in VN tests by a plaque reduction method on chick embryo fibroblast monolayers using the Nakayama-NIH strain of JE virus that had been multiply passaged in mouse brain as has been reported (Shortridge et al., 1974). Basically, infected mouse brain containing 50 to 150 PFU in the final inoculum in a 0.5% lactalbumin/Hank's medium/5% calf serum mixture was added to dilutions of serum, held at 37°C for 90 minutes and then allowed to adsorb for a further 90 minutes onto the cell sheet before adding an agar overlay of 0.5% yeast extract and 0.5% lactalbumin. A second overlay containing neutral red (final, 1:36,000) in Earle's saline was added after 2 days and plaques read after a further 1 to 2 days. The VN titer was expressed as the serum dilution at which 50% plaque reduction occurred.

Density gradient centrifugation

This was carried out in linear sucrose gradients (35%-7% w/v in 0.15 M NaCl in 0.01 M Tris-HCl pH 7.5) and the gradients centrifuged in 5 ml swinging bucket rotors at 200,000 × g for 7 hours (Shortridge et al., 1974). A marker A/Asia/57 (H2N2) rabbit antiserum containing 7S IgG and 12S NSI for this virus was centrifuged in parallel (Shortridge and Biddle, 1970). The gradient fractions for JE virus HAI titrations were mixed with a protein concentrate (bovine serum albumin, Dade Reagents Inc., Miami, Florida; human serum α_2-macroglobulin, Hyland Laboratories, Costa Mesa, California; human fibrinogen, BDH, Poole, England) to give a final protein concentration of approxi-

mately 7 gm/100 ml before treating with kaolin in order to circumvent non-specific adsorption of antibody in ·those gradient fractions low in total protein (Shortridge et al., 1974).

RESULTS

JE Virus Antibody Distribution and Content

All sera contained (i) natural agglutinins to low titer for gander cells and (ii) HAI activity for JE virus in the titer range 10-2560. Treatment of the sera with kaolin to remove hemagglutination non-specific inhibitor (NSI) resulted in complete removal of inhibitory activity from 30 out of 75 samples while the remaining 45 were of titer 10-240 indicating the presence of presumptive JE virus antibody. However, in the VN test, which is considered more reliable for arbovirus antibody detection, 58 samples were antibody positive with a titer of 10 or greater, and of these, lower titer samples predominated which was not so evident for the corresponding HAI positive samples (Fig. 1). The mean HAI and VN titers of these seropositive samples were 45 and 58, respectively. When considered on a seasonal basis there were no significant differences in HAI and VN titers in the spring/summer or autumn/winter seasons (Table 1). A low correlation coefficient of 0.1 was calculated for the two tests; similar findings were made with the cobra (Shortridge et al., 1974).
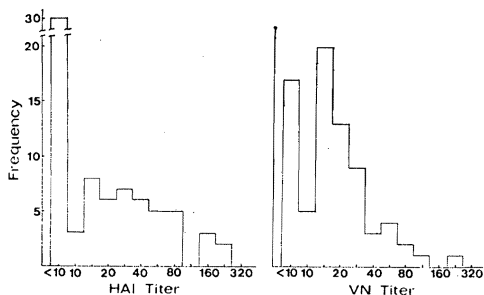

Fig. 1—Frequency distribution of JE virus HAI (after kaolin treatment) and VN antibody activities in turtle plasma.

An overall breakdown of the findings shows that 77% and 60% of the samples were antibody positive by VN and HAI tests, respectively, while 41% were positive by the one or the other and 11% were negative by both tests (Table 2).

Significance of NSI in Neutralization of JE Virus Infectivity

The validity of VN tests for detecting arbovirus antibody depends largely on the NSI not having neutralizing activity. Studies on human and reptile, viz. cobra, sera have shown that JE virus NSI activity resides in the lipoprotein fraction (Shortridge and Ho, 1974; Shortridge and Yip, in preparation). Accordingly, pools derived from 3 like JE virus VN positive or negative samples were submitted to preparative high speed centrifugation (Biddle and Shortridge, 1967). The upper lipoprotein layers were aspirated and then examined for their ability to neutralize JE virus infectivity. In two experiments performed on VN positive plasma, the lipoproteins derived from one pool lacked neutralizing activity while those from the other were of titer 15, but this activity was destroyed by heating at 56°C/30 minutes, the customary procedure used for treating serum before the VN test. Lipoproteins derived from the VN negative sera lacked neutralizing activity.

In separate experiments, the plasma from 6 sea snakes of the species *H. cyanocynctus*, was found to have HAI titers of 240-320 which reduced to 10 on kaolin treatment. When one of these samples was examined in the VN test, it lacked neutralizing activity.

Rate Zonal Centrifugation

Previous studies on the cobra showed this reptile to exhibit marked seasonal differences in mean HAI titer (Table 1) which were found to be associated with differences in distribution of specific immunoglobulins (Shortridge

Table 1

Titer characteristics of turtle plasma HAI*,** and VN** JE virus antibody activities over Spring/Summer (April-September) and Autumn/Winter (October-March) seasons compared with similar data for the cobra.

Animal	Characteristic	Seasonal titers	
		Spring/Summer	Autumn/Winter
Turtle	Mean HAI titer	35 ± 49 (32)***	29 ± 50 (43)
	HAI titer range	10 — 240	10 — 240
(T. sinensis)	Mean VN titer	29 ± 44 (32)	18 ± 16 (43)
	VN titer range	11 — 238	12 — 75
Cobra****	Mean HAI titer	712 ± 651 (30)	127 ± 181(50)
	HAI titer range	10 — 2560	10 — 640
(N. naja)	Mean VN titer	49 ± 23 (30)	15 ± 16 (50)
	VN titer range	10 — 100	12 — 84

* After kaolin treatment.
** A positive response is a titer of 10 or greater.
  Titers < 10 were scored as zero for determining mean titers.
*** Figures in brackets denote the number of samples studied by either test for each season.
**** Data from Shortridge et al., (1974).

Table 2

Summarized results of HAI*,** and VN** tests for JE virus antibody in the plasma of seventy-five turtles collected over a one year period.

Test or test combination	Number	Percentage
Total HAI pos.	45	60
Total VN pos.	58	77
HAI pos.; VN pos.	36	48
HAI pos.; VN neg.	9	12 ⎫
HAI neg.; VN pos.	22	29 ⎬ 41
HAI neg.; VN neg.	8	11 ⎭

* After kaolin treatment.
** A positive response is a titer of 10 or greater for each sample.

et al., 1974). As the HAI titer characteristics of the turtle are quite different, their plasma was submitted to rate zonal centrifugation under conditions previously used for studying the cobra in order to estimate the sedimentation properties of HAI JE virus specific immunoglobulins.

Five turtle plasma samples from June (3)/ July (2) and December (1)/ January (4), the middle months of the spring/summer and autumn/winter seasons, respectively, were pooled according to season and centrifuged as described under MATERIALS AND METHODS. Trypsinized human group "O" erythrocytes were used in the HAI titration of the gradient fractions. Inhibitory activity extended one-half to two-thirds from the top of the gradient (Fig. 2A and B). Treatment of the fractions with kaolin to remove NSI resulted in the removal of the bulk of this activity leaving peaks of residual antibody at Fraction 17 for plasma from both autumn/ winter and spring/summer seasons. The antiserum marker moved to a similar position (Fig. 2C) thus giving the JE virus specific antibody an interpolated sedimentation coefficient of 7S. The broad spread of NSI activity is in harmony with findings made on cobra plasma suggesting that this activity is associated with lipoproteins, in particular, the high density lipoprotein fraction (Shortridge et al., 1974).
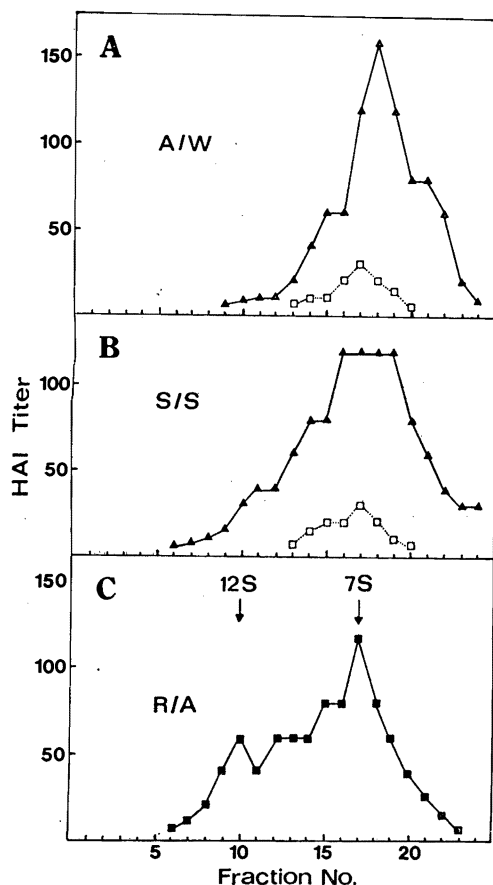
Fig. 2—Density gradient centrifugation of pools of five plasma samples collected from turtles during the middle of the autumn/winter (A/W) and spring/summer (S/S) seasons and a rabbit antiserum against A/Asia/57 influenza virus (R/A) containing markers of established sedimentation coefficient. All fractions were titrated in the HAI test—A and B, untreated (▲——▲) and kaolin treated (□.......□) against JE virus; C, untreated (■——■) against A/Asia/57 virus.

Interpolation of the 7S (antibody) and 12S (NSI) marker positions in the centrifuged rabbit antiserum indicated that JE virus specific 18S macroglobulin antibody, if present, would have been detected around Fractions 1 and 2. Similarly, a 17S component, as was found in autumn/winter cobra plasma (Shortridge et al., 1974) would be present as a peak at Fraction 3. As no HAI activity i.e. < 10, was detected in any of the bottom fractions it can be

safely concluded that macroglobulin antibody was not present at a significant level in the samples examined.

DISCUSSION

Arbovirus non-specific inhibitory (NSI) activity in vertebrate sera appears to be confined to the lipoprotein fraction (Bidwell and Mills, 1968; Shortridge and Ho, 1974; Shortridge and Yip, in preparation). Kaolin treatment of reptile serum in contrast to acetone treatment (Yuill et al., 1971) appears effective in removing this activity (Bidwell and Mills, 1968) suggesting that residual HAI activity is due to antibody. Similar considerations would appear to apply to turtle plasma studied here for which a kaolin insensitive 7S component was found to be associated with residual HAI activity. In addition, plasma from a sca-snake, an animal most unlikely to have arbovirus antibody, was VN negative indicating that reptile lipoproteins are devoid of significant VN activity as was confirmed using partially purified turtle lipoproteins. Thus it may be reasonably presumed that the high level of arbovirus seropositivity of turtle plasma detected with JE virus is due to antibody rather than NSI. The possibility that some undefined NSI may participate in the HAI reaction to low titer cannot be completely excluded.

JE virus is the only known group B arbovirus in Hong Kong, an area which is contiguous with the southern Chinese provinces where the soft-shelled fresh water turtle is most common (Pope, 1935). It is noteworthy, that JE virus is the only arbovirus in China for which documentation is available (Huang, 1972). The serological findings of this study viewed against the above background strongly suggest that the inhibitory activity detected in T. sinensis is the result of infection by JE virus.

A high incidence of JE virus infection (70% by VN and 85% by HAI) has been recorded for the cobra (Shortridge *et al.*, 1974) and the same would appear to apply to the turtle where the incidence was 77% and 60% by VN and HAI tests, respectively. These incidence levels are based on titers of 10 and above being taken as significant. As the VN negative sea snake plasma had a low HAI titer of 10 after kaolin treatment, a titer of 20 might be more acceptable as an antibody baseline HAI titer and consequently this, in the case of the turtle plasma, would reduce the positive HAI incidence level to 41%. Since IgM antibody was not detected in pools of samples from spring/summer and autumn/winter seasons, it would seem unlikely that the lower level of HAI reactivity could be due to its concommitant removal with NSI during kaolin treatment (Schmidt *et al.*, 1971).

It is worth noting at this juncture that when the positive results of HAI and VN tests are jointly considered, the incidence of JE virus infection is 89% (Table 2) which compares with an incidence of 91% obtained in a complementary study on the cobra (Shortridge *et al.*, 1974). However, it should be noted that 12% and 29% of the samples would have been reported negative had VN and HAI tests, respectively, been solely used. These findings underline again the importance of the VN test in investigative work of this nature.

The ability of reptiles to mount a secondary anamnestic response is controversial. Macroglobulin and 7S antibodies have been reported for the painted turtle (Grey, 1963) and the European pond tortoise (Lykakis, 1968) while more recent studies have shown that the lizard *Tiliqua rugosa* is capable of a secondary response to *Salmonella typhimurium* which is associated with macroglobulin antibody (Wetherall and Turner, 1972). No macroglobulin HAI antibody was detected in *T. sinensis* although these turtles must have been bitten frequently particularly during the mosquito

breeding cycle. In addition, no significant seasonal changes in the 7S HAI and VN antibody levels were detected in spite of the fact that the turtle, like other cold-blooded animals undergoes considerable physiological changes over a one year period. These changes in the snake, genus *Thamnopsis*, have been shown to alter appreciably the circulating antibody content and the level of WEE viraemia (Prior and Agnew, 1971). The behaviour of *T. sinensis* is in marked contrast to that of the cobra, *N. naja*, which manifested (i) considerably higher JE virus HAI and VN titers in spring/summer and (ii) lower titers in the autumn/winter seasons, the levels of which were similar to those seen throughout the year for *T. sinensis*. HAI reactivity in the former seasonal group was associated with 7S antibody and in the latter, with both macroglobulin and 7S antibodies (Shortridge *et al.*, 1974). The geographical distribution of *T. sinensis* and *N. naja* in China overlap (Pope, 1935) and both animals are probably subject to the same vector. The reason for the marked differences in antibody response to JE virus is unclear.

Combined virus isolation, serological studies and experimental infection have shown that up to nine different turtle species may be infected by a number of arboviruses (see review by Hoff and Trainer, 1973). The findings of this study appear to extend the range of turtles to *T. sinensis*. The only other turtles reported to be infected by a group B arbovirus are *Chrysemys p. picta* and *Chelydra serpentina*, both by St. Louis encephalitis in the United States (Whitney *et al.*, 1968; Goldfield and Sussman, 1964). *T. sinensis* is found in China and is abundant in shallow rivers, lakes and muddy areas (Pope, 1935). Japanese encephalitis in China is reported to be transmitted chiefly by *Culex pipiens* var. *pallens* and *Anopheles hyrcanus* var. *sinensis* [(A. sinensis) Wiedemann] (Huang, 1972) but whether or not these particular mosquitoes

are associated with *T. sinensis* is not known. The finding by Hayes (1961) that *Culex pipiens* is associated with turtles suggests a similar situation may apply to this mosquito and *T. sinensis*. Experimentally infected *Culex fatigans* has been reported capable of transmitting JE virus transovarially (Sum, 1956) but it is unclear as to whether the vectors given above may behave in like manner.

T. sinensis is widely distributed along the coast of China, especially in Hainan, Kwangtung and Taiwan. It also extends to Hopei and Jehol in the north, and westward to Kwangsi (where the turtles used in this study came from), Yunnan and Szechwan (Pope, 1935). As Japanese encephalitis is reported to be widely distributed throughout China (Huang, 1972), the limited geographical distribution of *T. sinensis* would limit any functional role it may have in the maintenance cycle of JE virus. The snake, genus *Thamnopsis*, has been suggested to be a possible overwintering host for WEE virus, (Thomas and Eklund, 1960 and 1962) and a similar relationship based on JE virus antibody characteristics was recently put forward by Shortridge *et al.*, (1974) for the cobra, *N. naja*. The apparent year long consistency of JE virus specific 7S IgG suggests this may be less likely with *T. sinensis*.

Arbovirus serology is obviously subject to a number of vagaries; this is especially so for the lower vertebrates. Nonetheless, the findings of this study provide further evidence of association between poikilothermic hosts and certain arboviruses, in this case the human and animal pathogen, Japanese encephalitis virus.

SUMMARY

This study was undertaken to examine further the natural infection of poikilothermic animals e.g. turtles, to Japanese encephalitis (JE) virus. Plasma samples from 75 soft-shelled fresh water turtles (*Trionyx sinensis* Wiegman) from China were examined in virus neutralization (VN) and hemagglutination inhibition (HAI) tests for the presence of specific antibody. The total incidence of antibody detected by either test to a titer of 10 or greater was 89% while 77% and 60% were positive by VN and HAI tests, respectively. Forty-one per cent were jointly positive by both tests. Mean HAI and VN titers were similar and showed no obvious differences between spring/summer and autumn/winter seasons. The HAI reactivity was associated with a 7S component for both seasons. The significance of this inhibition in the serology of poikilothermic hosts and the possible behaviour of *T. sinensis* in the natural history of JE virus is briefly considered.

ACKNOWLEDGEMENTS

REFERENCES

BIDDLE, F. and SHORTRIDGE, K.F., (1967). Immunological cross-reactions of influenza virus inhibitors. *Brit. J. Exp. Path.*, *48* : 285.

BIDWELL, D.E. and MILLS, G.L., (1968). Serum non-specific inhibitors of arbovirus haemagglutination. *J. Comp. Path.*, *78* : 469.

BUESCHER, E.L. and SCHERER, W.F., (1959). Ecologic studies of Japanese encephalitis virus in Japan. IX. Epidemic correlations and conclusions. *Amer. J. Trop. Med. Hyg.*, *8* : 719.

CLARKE, D.H. and CASALS, J., (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Amer. J. Trop. Med. Hyg.*, *7* : 561.

DETELS, R., CATES, M.D., CROSS, J.H., IRVING, G.S. and WATTEN, R.H., (1970).

Ecology of Japanese encephalitis virus on Taiwan in 1968. *Amer. J. Trop. Med. Hyg.*, *19* : 716.

DOI, R., OYA, A. and TELFORD, S.R., (1968). A preliminary report on infection of the lizard, *Takydromus tachydromoides*, with Japanese encephalitis virus. *Jap. J. Med. Sci. Biol.*, *21* : 205.

GOLDFIELD, M. and SUSSMAN, O., (1964). Ecologic studies of eastern equine and western equine encephalitis viruses in New Jersey (non-avian vetebrate studies). Report presented at Center of Disease Control, Atlanta, Ga. August 6, 1964.

GREY, H.M., (1963). Phylogeny of the immune response. Studies on some physical, chemical and serologic characteristics of antibody produced in the turtle. *J. Immun.*, *91* : 819.

HAYES, R.O., (1961). Host preferences of *Culiseta melanura* and allied mosquitoes. *Mosq. News*, *21* : 179.

HOFF, G. and TRAINER, D.O., (1973). Arboviruses in reptiles: isolation of a Bunyamwera group virus from a naturally infected turtle. *J. Herpet.*, *7* : 55.

HUANG, K.Y., (1972). Medicine and Public Health in the People's Republic of China. Ed. J.R. Quinn, DHEW Publication No. (NIH) 72-67. p. 246.

HURLBUT, H.S., (1964). The pig-mosquito cycle of Japanese encephalitis virus in Taiwan. *J. Med. Entom.*, *1* : 301.

KONNO, J., ENDO, K., AGATSUMA, H. and ISHIDA, N., (1966). Cyclic outbreaks of Japanese encephalitis among pigs and humans. *Amer. J. Epidem.*, *84* : 292.

LEE, H.W., (1968). Multiplication and antibody formation of Japanese encephalitis virus in snakes. II. Proliferation of the virus. *Seoul J. Med.*, *9* : 157.

LEE, H.W., MIN, B.W. and LIM, Y.W., (1972). Isolation and serologic studies of Japanese encephalitis virus from snakes in Korea. *J. Korean Med. Ass.*, *15* : 69.

LYKAKIS, J.J., (1968). Immunoglobulin production in the European pond tortoise, *Emys orbicularis*, immunized with serum protein antigens. *Immunology*, *14* : 799.

MIFUNE, K., SHICHIJO, A., UEDA, Y., SUENAGA, O. and MYAGI, I., (1969). Low susceptibility of common snakes in Japan to Japanese encephalitis virus. *Trop. Med.*, *11* : 27.

POPE, C.H., (1935). The Reptiles of China. *In* Natural History of Central Asia. Vol. X. *Ed.* C.A. Reeds. The American Museum of Natural History, New York. pp. 59-63.

PRIOR, M.G. and AGNEW, R.M., (1971). Antibody against western equine encephalitis virus occurring in the serum of garter snakes (Colubridae : *Thamnopsis*) in Saskatchewan. *Canad. J. Comp. Path.*, *35* : 40.

SCHMIDT, N., GEE, P.S., DENNIS, J. and LENNETTE, E.H., (1971). Enzymes produced by a *Pseudomonas* species which inactivate inhibitors of certain viral hemagglutinins II. Effect of a proteinase and phospholipase C on viral hemagglutinin inhibitors present in human sera. *J. Immun.*, *106* : 1615.

SHORTRIDGE, K.F. and BIDDLE, F., (1970). The proteins of adenovirus type 5. *Arch. Ges. Virusforsch.*, *29* : 1.

SHORTRIDGE, K.F. and HU, L.Y., (1974). Trypsinized human group O erythrocytes as an alternative hemagglutinating agent for Japanese encephalitis virus. *Appl. Microbiol.*, *27* : 653.

SHORTRIDGE, K.F. and HO, W.K.K., (1974). Human serum lipoproteins as inhibitors of haemagglutination for selected togaviruses. *J. Gen. Virol.*, *23* . 113.

SHORTRIDGE, K.F., NG, M.H., OYA, A., KOBAYASHI, M., MUNRO, R., WONG, F. and

LANCE, V., (1974). Arbovirus infections in reptiles : immunological evidence for a high incidence of Japanese encephalitis virus in the cobra, *Naja naja*. *Trans. Roy. Soc. Trop. Med. Hyg.*, *68* : 454.

SUM, TING-HUNG, (1956). Experimental study of transovarial passage of Japanese B virus in mosquitoes. *Second Conference of the Chinese Microbiology Society.* Peking. p. 191.

THOMAS, L.A. and EKLUND, C.M., (1960). Overwintering of western equine encephalomyelitis virus in experimentally infected garter snakes and transmission to mosquitoes. *Proc. Soc. Exp. Biol. Med.*, *105* : 52.

THOMAS, L.A. and EKLUND, C.M., (1962). Overwintering of western encephalomyelitis virus in garter snakes experimentally infected with *Culex tarsalis*. *Proc. Soc. Exp. Biol. Med.*, *109* : 421.

WETHERALL, J.D. and TURNER, K.J., (1972). Immune response of the lizard *Tiliqua rugosa*. *Aust. J. Exp. Biol. Med. Sci.*, *50* : 79.

WHITNEY, E., JAMNBACK, J., MEANS, R.G. and WATTHEW, T.H., (1968). Arthropod-borne virus survey in St. Lawrence County, New York: Arbovirus reactivity in serum from amphibians, reptiles, birds and mammals. *Amer. J. Trop. Med. Hyg.*, *17* : 645.

YUILL, T.M., BRANDT, W.E. and BUESCHER, E.L., (1971). Nonspecific arbovirus hemagglutination inhibitors in turtle sera. *J. Immun.*, *106* : 1413.