Ntaya Virus. A Hitherto Unknown Agent Isolated from Mosquitoes Collected in Uganda.* (18700)

K. C. SMITHBURN AND A. J. HADDOW.

From the Laboratories, International Health Division, Rockefeller Foundation, New York City, and the Virus Research Institute, Entebbe, Uganda.

The purpose of this communication is to record the isolation in 1943 of a previously unknown neutrotropic virus which is now, along with other recently discovered viruses, being studied further in New York. The circumstances concerned with its isolation, together with subsequent observations, render it impossible to state its exact origin but it is believed that it was derived from wild mosquitoes. The name applied is in respect to the locality where the mosquitoes were caught, the Ntaya swamp in Bwamba County, Uganda Protectorate, British East Africa.

Ntaya virus was encountered during the course of coordinated laboratory and field investigations, the purpose of which was to discover the insects responsible for the transmission of yellow fever in extra-human cycles of natural infection. Yellow fever is enzootic among the wild primates of Bwamba County (Haddow, *et al.*)(1), and is also either endemic or repetitively epidemic among the human population of the area (Mahaffy, *et al.*)(2). The procedure in the attempts to discover the vector (or vectors) was to capture mosquitoes in selected localities, to classify them, and then to inoculate suspensions of them into susceptible laboratory animals (mice and rhesus monkeys) to test for the presence of virus. The methods which are effective for the isolation of yellow fever virus (Mahaffy, *et al.*)(2); Smithburn and Haddow(3); Smithburn, Haddow, and Lumsden(4), have also yielded several other viruses, 2 of which were previously known: Rift Valley fever (Smithburn, Haddow, and Gillett)(5), and Mengo (Dick, Smithburn, and Haddow)(6), the latter being identical with encephalomyocarditis, M. M. and Columbia S-K viruses (Dick)(7); Warren, Smadel, and Russ(8). Five other agents, each apparently hitherto unknown, were isolated in the same manner, including: Semliki Forest (Smithburn and Haddow)(9), Bunyamwera (Smithburn, Haddow, and Mahaffy)(10), Uganda S(11) and Zika(12) viruses, in addition to the Ntaya virus discussed here. The details of methods employed have been given in previously mentioned papers.

As has been stated elsewhere (Haddow)(13), the Bwamba lowlands include a large tract of uninhabited primeval forest and a densely populated agricultural area. The latter is much cut up by deep valleys which contain relict belts and patches of primary

* The studies reported here were made in part at the Yellow Fever Research Institute, Entebbe, Uganda (presently the Virus Research Institute), then supported jointly by the Medical Department of the Government of Uganda Protectorate and the International Health Division of The Rockefeller Foundation, and in part in the laboratories of the International Health Division in New York.

1. Haddow, A. J., Smithburn, K. C., Mahaffy, A. F., and Bugher, J. C., 1947, *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1947, v40, 677.

2. Mahaffy, A. F., Smithburn, K. C., Jacobs, H. R., and Gillett, J. D., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1942, v36, 9.

3. Smithburn, K. C. and Haddow, A. J., *Am. J. Trop. Med.*, 1946, v26, 261.

4. Smithburn, K. C., Haddow, A. J., and Lumsden, W. H. R., *Ann. Trop. Med.*, 1949, v43, 74.

5. Smithburn, K. C., Haddow, A. J., and Gillett, J. D., *Brit. J. Exp. Path.*, 1948, v29, 107.

6. Dick, G. W. A., Smithburn, K. C., and Haddow, A. J., *Brit. J. Exp. Path.*, 1948, v29, 547.

7. Dick, G. W. A., *J. Immunol.*, 1949, v62, 375.

8. Warren, J., Smadel, J. E., and Russ, S. B., *J. Immunol.*, 1949, v62, 387.

9. Smithburn, K. C., Haddow, A. J., *J. Immunol.*, 1944, v49, 141.

10. Smithburn, K. C., Haddow, A. J., and Mahaffy, A. F., *Am. J. Trop. Med.*, 1946, v26, 189.

11. Dick, G. W. A., and Haddow, A. J., to be published.

12. Dick, G. W. A., Kitchen, S. F., and Haddow, A. J., to be published.

13. Haddow, A. J., *Proc. Zool. Soc. Lond.*, 1945, v115, 1.

forest, some of them continuous with the main Semliki Forest. These relict strips have survived mainly in localities unsuited to agricultural development. The Ntaya swamp is a marshy reach of the Manjuguja River, near its source, lying in a deep valley about 1½ miles southwest of Bundibugyo. The relict forest there in 1943 was very dense and tangled, its most characteristic feature being a predominance of old oil palms (*Elaeis*). It was very small in extent—not more than half a square mile in all—and had no connection with the main uninhabited forest, being surrounded on all sides by native plantations and dwellings. This forest has now almost entirely disappeared.

In the early months of 1943 Bwamba County was suffering an unusual period of drought, and the Ntaya swamp area was the only locality in which it was possible to collect mosquitoes in sufficient numbers to warrant a full-time catch. Moreover, the catch which was made yielded so few mosquitoes of species which were interesting or suspect as possible vectors of yellow fever that the usual procedure of inoculating highly suspect species separately was dispensed with. Instead, only the 120 *Eretmapodites* spp. and the 66 *Aedimorphus* spp. were treated separately. No pathogenic agent was recovered from these insects. All of the other mosquitoes taken in 5 successive days of catching were grouped for the inoculations. The latter lot, which presumably gave origin to the Ntaya virus, included:

Taeniorhynchus	
(Coquillettidia) pseudoconopas Theo.	4
Uranotaenia alboabdominalis Theo.	4
Theobaldia (Theomyia) fraseri Edw.	6
T. (C.) aurites Theo.	2
Aedes (Stegomyia) simpsoni Theo.†	1
A. (S.) apicoargenteus Theo.	12
A. (S.) africanus Theo.‡	3
A. (Dunnius) albomarginatus Newst.	2
Culex (Lutzia) tigripes group Theo.	31
C. (Culex) poicilipes Theo.	4
C. (C.) pruina Theo.	104
C. (C.) moucheti Ev.	271
Culex Ev. spp. indet. (about 12 species)	874
Total	1318

These 1318 mosquitoes were dispatched from the field station in Bwamba to the central laboratory at Entebbe. On arrival Feb. 22, 1943, all the living mosquitoes were chloroformed and ground up, together with 162 which had died en route, in 10% normal monkey serum in physiological saline. A portion of the supernate of the suspension was Seitz-filtered and inoculated intracerebrally into mice and the remaining unfiltered supernate was injected subcutaneously into normal rhesus monkey No. 276.

The monkey exhibited neither fever nor other signs of illness following inoculation. Of the 6 mice inoculated with the mosquito suspension 2 exhibited paralysis, 1 on the 7th and 1 on the 9th day, while the other 4 remained well. Brain passages were made from the 2 mice showing paralysis, both unfiltered and Seitz-filtered suspensions being used, with resulting successful transmission in each instance. One to 3 of 12 mice survived in each of the first 3 intracerebral passages, and there were occasional survivors through 11 passages, after which all the mice in the passage series died. Duplicate lines were successfully transmitted through 100 successive brain-to-brain passages in mice. Owing, doubtless, to adaptation to the brain of the white mouse as a host, there was gradual diminution of the average survival time of mice in the serial transfers. The average survival time of the 74 mice which succumbed in the 2d to 11th passages, disregarding 20 which survived, was 8.1 days; the average survival time among 101 mice of the 91st to 100th passages, in which there were no survivors, was 5.9 days. Seitz filtrates of

† The single specimen of *Aedes simpsoni* was originally determined as *A. metallicus* Edw. At a later date comparative material became available and it was found that what had been taken for this species was a rare variant of *A. simpsoni* with an aberrant scutal pattern (Haddow, *et al.*) (14). Had it been realized that this mosquito was a specimen of *A. simpsoni*, it would not have been included in the mixed batch, as this species was already known to be an important vector of yellow fever among human beings in Bwamba County.

‡ At this time *Aedes africanus* was not particularly suspected of being a forest vector of yellow fever, as it eventually proved to be (Smithburn, Haddow, and Lumsden) (4); had it been, these specimens would have been inoculated separately.

14. Haddow, A. J., van Someren, E. C. C., Lumsden, W. H. R., Harper, J. O., and Gillett, J. D., *Bull. Ent. Res.*, in press.

brain were occasionally used for passage, invariably with success, and with no noteworthy prolongation of survival time. The agent was also found to be filterable through all grades of Berkefeld filters.

Rhesus No. 276, which received the mosquito suspension, exhibited no neutralizing antibody in its serum either 3 weeks or 8 months following the inoculation of the mosquito suspension. From this evidence one might suspect that the virus did not derive from the mosquitoes. However, 2 subinoculations of 10% mouse brain from the paralyzed mice of the first passage were made to another normal rhesus, No. 282, and this animal likewise failed to develop antibody as a result. The latter was found to be capable of producing antibody when it developed humoral immunity following 2 further inoculations of concentrated mouse brain passage virus. It appeared, therefore, that the Ntaya virus is neither highly pathogenic nor strongly antigenic for rhesus monkeys, in view of which the failure to demonstrate antibody in rhesus No. 276 does not preclude the derivation of the virus from the mosquitoes. No agent similar to Ntaya virus was ever before or since encountered in the stock mice at Entebbe. It is therefore our opinion that the virus was, in fact, derived from the Ntaya mosquitoes, although proof of this point is impossible to obtain.

Properties of Ntaya virus. Comprehensive studies of this agent have not yet been made, owing in part to uncertainty as to the origin of the virus up to the time that monkey No. 282 finally developed neutralizing antibody. The data available concerning it are presented as a preliminary study and further information will be forthcoming when studies now in progress are completed on this and other viruses recently isolated in the yellow fever laboratories in East and West Africa, Colombia and Brazil.

Pathogenic properties in mice. After an incubation period of 3 to 7 days, depending on the passage level and the dilution of the inoculum, mice inoculated intracerebrally with mouse brain Ntaya virus show the first signs of illness. Many of the mice show paralysis, first of the hind quarters, and later of the fore, while these and others showing no paralysis exhibit a roughening of the pelage, mild tremors, and occasionally convulsions. About 48 hours usually elapse between the onset of symptoms and death, prior to which the majority of mice are either paralyzed or prostrate. Deaths occur between the 5th and 14th days, depending on the passage level and the dose of virus inoculated. Intranasal and intraperitoneal inoculation of the virus into mice did not cause illness, the intraperitoneal inoculations proving harmless even after intracerebral inoculation of an irritant, sterile starch solution, as practiced in the intraperitoneal yellow fever protection test (Sawyer and Lloyd(15). In the earliest passages the titers of virus in the brains of inoculated mice were only 10^{-4} to 10^{-5}. With continued passage there was a moderate increase in potency, titers at the 56th passage reaching 10^{-6} to $10^{-6.5}$. Titers as high as 10^{-7} have not been observed.

Pathogenicity for rhesus monkeys and hamsters. Other than the aforementioned two monkeys, only one additional rhesus has been inoculated with Ntaya virus. Rhesus M4898 was inoculated subcutaneously in New York with 8400 LD_{50} of virus, failed to show either fever or other signs of illness, and, like monkeys Nos. 276 and 282 in Uganda, developed little or no neutralizing antibody. Rhesus M4898 received a further 3 injections of concentrated virus, to which there was no clinical response, yet the animal, like rhesus No. 282, then exhibited potent neutralizing antibody in its serum. Young Syrian hamsters inoculated intracerebrally or subcutaneously exhibited no clinical reactions to the virus. One hamster inoculated intracerebrally with 205 LD_{50} developed little or no antibody as a result of the inoculation; another inoculated subcutaneously with 6800 LD_{50} developed antibody in moderate titer. The latter may indicate that hamsters are more susceptible to the virus than rhesus monkeys. The pathogenicity of the virus for other species of animals has not been tested.

Preservation of the agent. Ntaya virus

15. Sawyer, W. A. and Lloyd, W., *J. Exp. Med.*, 1931, v54, 533.

TABLE I.
Intracerebral Neutralization Test in Mice Showing the Development of Antibody in Rhesus No. 282 after 4, but not after 2, Injections of Concentrated Virus Suspension.

Serum	No. injections of virus	Interval after last inj., days	Titer of virus, log	LD_{50} neutralized, log
Normal rhesus pool	0	—	6.3	—
No. 282 Pre-inoculation	0	—	6.6	—
" " Mar. 15	2	12	6.15	0.45
" " May 18	4	12	3.4	3.2
" " Oct. 25	4	172	3.5	3.1

may readily be preserved by lyophilization. Eighth passage virus was titrated before and 20 days after desiccation, with the following results:

Titer of original suspension $10^{-6.31}$
Titer 20 days after desiccation $10^{-5.94}$

The aforementioned desiccated virus was still potent 3½ years later, after shipment without refrigeration to New York. A desiccated lot of 13th passage virus prepared in New York showed no significant decline in potency within 15 months. The virus may also be preserved, at least for short periods, by storage of the mouse brain suspension in the frozen state, either at –25°C in a mechanical refrigerator, or in the solid CO_2 cabinet.

Immunological reactions. When a suitably potent immune serum was finally obtained, it was found possible to perform intracerebral neutralization tests in mice essentially according to the method developed by Theiler(16) for the intracerebral yellow fever neutralization test. 0.3 cc portions of suitable dilutions of fresh mouse passage or desiccated virus are added to 0.3 cc portions of whole sera, the mixtures are incubated 2 hours at 37°C and inoculated intracerebrally in 0.03 cc quantities into susceptible Swiss mice. Results of the test showing the development of antibody in rhesus No. 282 are shown in Table I.

It is noteworthy that the immunity acquired by rhesus No. 282 was retained from May, 1943, without any further known contact with Ntaya virus, and serum taken from the animal on September 19, 1949, still contained abundant antibody. Thus, although a potent immune response is not easily evoked, once the antibody develops it is well retained.

Immediately after its isolation Ntaya virus was tested against yellow fever immune serum, with negative results. Recently, reciprocal cross neutralization tests were done with Ntaya virus and a number of other neurotropic viruses without any strong cross reaction being observed. The agents tested in the latter studies included: Bwamba fever, West Nile, Semliki Forest, Bunyamwera, Mengo, Uganda S and Zika viruses, all isolated in Uganda; Kumba virus, isolated in the Cameroons; Anopheles A, Anopheles B, and Wyeomyia viruses, isolated in Colombia (Roca Garcia)(17); Ilhéus virus, isolated in Brazil (Laemmert and Hughes)(18); and 10 previously known agents—Western, Eastern and Venezuelan equine encephalomyelitis, St. Louis, Japanese B and Russian spring-summer encephalitis, louping ill, encephalomyocarditis, Dengue (Hawaiian strain) and yellow fever viruses.

In an effort to ascertain whether or not Ntaya virus attacks man, the sera of 43 persons residing near the Ntaya swamp were tested for antibody against the virus. None of these exhibited any neutralizing power. Further studies of this nature are anticipated but have not yet been made. At this time, therefore, its natural hosts remain unknown.

Summary. A filterable neurotropic virus was isolated from mice inoculated intracerebrally with a mixed lot of mosquitoes captured in western Uganda. The agent is believed to be hitherto unknown and has been named Ntaya virus in respect of the locality in which the mosquitoes were caught. Certain of its pathogenic and immunological properties are discussed.

16. Theiler, M., *Ann. Trop. Med.*, 1933, v27, 57.
17. Roca Garcia M., *J. Infect. Dis.*, 1944, v75, 160.
18. Laemmert, H. W., Jr., and Hughes, T. P., *J. Immunol.*, 1947, v55, 1.

Received April 17, 1951. P.S.E.B.M., 1951, v77.