ORUNGO (UgMP 359) VIRUS: A HITHERTO UNDESCRIBED VIRUS, BIOCHEMICAL, BIOPHYSICAL AND EPIDEMIOLOGICAL STUDIES

BY

OYEWALE TOMORI
D.V.M. (Zaria)
A thesis in the VIRUS RESEARCH LABORATORY

Submitted to the Faculty of Medicine in partial fulfilment of the requirements for the degree of

DOCTOR OF PHYLOSOPHY

of the

UNIVERSITY OF IBADAN

4th June, 1976.

Frontispiece. Orungo virus particles with associated filaments. X111,100

DEDICATED

TO THE MEMORY OF

OLADEJI OYEWALE TOMORI

THE SON I DID NOT KNOW

BECAUSE

I WAS IN SEARCH OF

ORUNGO VIRUS

ABSTRACT

Orungo virus, a hitherto undescribed virus, first isolated from a pool of Anopheles mosquitoes caught off human bait in Uganda was studied by biochemical, biophysical and sero-epidemiological methods. These studies were conducted to classify Orungo virus, determine antigenic differences between Orungo virus strains, and determine the host range and extent of infection of Orungo virus in man and animals in Nigeria. The techniques employed included reactions of Orungo virus to physical and chemical agents, electron microscopy, to determine the ultrastructure of the virus, neutralization tests in new-born-mice, complement fixation, hemagglutination and agar gel precipitation tests. Other techniques used were plaque formation in tissue culture, experimental infection of laboratory animals and transmission studies with arthropods.

Orungo virus, with a virus particle size of 63nm and an icosahedral capsid construction was found to be similar in all details of morphology and morphogenesis to the orbiviruses - a group of viruses which are serologically unrelated but morphologically and morphogenetically identical. Orungo virus shares with the orbiviruses, the common property of relative stability to lipid solvents and sodium desoxycholate, lability at pH 3.0 and lack of antigenic relationship to any of the major serologic

group A, B and Bunyamwera. Orungo virus was found to be thermolabile at 56°C, sensitive to UV irradiation and treatment with BPL and formalin. The host range susceptibility include the Swiss albino mice, hamsters, lambs and rabbits. Sparrows and day old chicks neither circulated virus nor developed antiboty following inoculation with Orungo virus.

Orungo virus multiplies with resultant CPE in Vero and BHK-21 cell lines, but not in Aedes albopictus cell cultures. Experimental transmission of Orungo virus was achieved with Aedes albopictus and Aedes aegypti mosquitoes inoculated by the intrathoracic route.

Orungo virus does not show hemagglutinating activity, however by CF, and N tests varying degrees of differences were demonstrated between the Orungo virus strains. In AGD tests, there was a complete line of identity with all the strains. Strain H60974 previously reported as a strain of Orungo virus was found to be Tataguine virus.

Neutralizing antibodies to Orungo virus were detected in the sera of man and animals collected from different parts of Nigeria. There was an increasing trend in prevalence rate from the wet forested area to the drier savannah regions. In addition the prevalence of antibody increased with age.

The commonly described symptoms of Orungo virus infection are mild fever of short duration, myalgia, headache and occasional weakness of the lower extremities.

ACKNOWLEDGEMENTS

The story of Orungo virus is one of international co-operation.

My sincere and profound gratitude first of all goes to Professor A. Fabiyi, the Director of the Virus Research Laboratory who supervised the investigations on Orungo virus.

Dr. G. B. Kirya, who as the Head of the Department of Arbovirology of the East African Virus Research Institute gave his very strong support for the study of this virus, deserves no less an appreciation. He, it was, who gave the initial courage to embark on this project.

In the process of studying Orungo virus, I had the priviledge and the rare opportunity of working with eminent scientists (too numerous to list) in three of the best research laboratories in the United States of America. I wish to place on record my heart-felt gratitude to all my friends and colleagues at the Special Pathogens Division and the Viropathology Division of the Centre for Disease Control (CDC) Atlanta, Georgia; the Vector Borne Diseases Division of the CDC at Fort Collins, Colorado; and the Yale Arbovirus Research Unit of the Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut, U.S.A. They gave so much of their time and energy to help someone who was particularly in a hurry. To my colleagues

in the University of Ibadan and the Virus Research Laboratory in particular who sacrificed time and material to see the studies on Orungo virus come to fruition, I express a sincere gratitude.

Without a happy home to return to, or a tender loving care to blunt the sharp edges of frustration and disappointment of research, I might not have completed these studies. To dear Omowumi, my wife, and Olanrewaju, my son, you both told me it was worth all the while. Thank you both so very much. I just wonder what I would have done without you both.

My sincere gratitude to Dr. P. Tukei, of the East African Virus Research Institute, Entebbe, Uganda who consented to the use of Orungo, as the name for the virus under study, otherwise these would have been the studies of a nameless virus.

Under a coating of modesty, I wish to pat myself on the back, for a job well done, just in case no-one remembers my own contributions.

I certify that this work was carried out by Dr. Oyewale Tomori in the Virus Research Laboratory, University of Ibadan.

(Supervisor)

Akinyele Fabiyi, B.Sc. (Syr.),
M.Sc. (Wash.), and Ph.D. (Penn.)
Research Professor of Virology
Virus Research Laboratory,
University of Ibadan,
Ibadan, Nigeria.

TABLE OF CONTENTS

Pages

LIST OF FIGURES

Pages

LIST OF PLATES

Pages

LIST OF TABLES

Pages

ABBREVIATIONS

AN	=	prefix for virus isolate from animals
AR	=	prefix for virus isolated from arthropods
BABS	=	bovine albumin in borate saline
BA/PS	=	bovine albumin diluent with penicillin and streptomycin
BPL	=	betapropiolactone
BSS	=	balanced salt solution
CF	=	complement fixation
dex	=	equivalent to logarithm to base 10
EAVRI	=	East African Virus Research Institute
H	=	prefix for virus isolate from human
HA	=	haemagglutination
HAI/HI	=	haemagglutination-inhibition
IMAF	=	immune mouse ascitic fluid
IC	=	intracerebral
IP	=	intraperitoneal
IV	=	intravenous
LNI	=	logarithm of neutralizing index
No.	=	number
p.i.	=	post inoculation
SC	=	subcutaneous
r.p.m.	=	revolutions per minute
Y.A.R.U.	=	Yale Arbovirus Research Unit.

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CHAPTER 1

INTRODUCTION

The Virus Research Laboratory in Ibadan was established in 1964 principally for the following objectives:

(1) To determine the incidence and prevalence of arboviruses in Nigeria.

(2) To learn the significance of such viruses to man and domestic animals.

(3) To study the epidemiology of those viruses which play significant role in the health and economics of man and animals.

(4) To consider the application of control measures based upon the knowledge obtained in pursuing these objectives.

Between 1964 and 1971 over sixty different types of viruses were isolated from man, animals and arthropods. Some of these viruses were isolated for the first time in the world. The groups, names and Ibadan prototype numbers of the viruses isolated in Nigeria are shown in Table 1. It would therefore appear that to a large extent, the first objective for establishing the Virus Research Laboratory had been achieved.

However, apart from what was known about the already established viruses such as yellow fever, dengue and chikungunya, very little information was available in respect of the new viruses isolated in

Nigeria. For viruses such as Sabo, Kotonkan or Igbo-Ora, no information was available as regards their significance to man and domestic animals nor was any information available as to the epidemiology of the viruses in Nigeria, not to mention application of control measures for such virus infections. In addition, more than 7 viruses had not even been classified as to virus group. In short, the other three objectives for establishing the Virus Research Laboratory were still to be achieved. The present studies on the biophysical, biochemical and epidemiological characteristics of the virus originally designated as UgMT 359, Williams et al. (1962), but now known as Orungo virus, Tomori (1976) are the beginning of attempts at the realisation of the other objectives for establishing the Virus Research Laboratory.

Orungo virus was first isolated from a pool of Anopheles mosquitoes caught on human bait at Orungo, Teso District of Uganda (Williams et al. op cit.). However, apart from this original isolate, no other strains of the virus has to-date been isolated in Uganda from any of the thousands of mosquito collection and other specimens processed for virus isolation. Moreover, serological surveys carried out in Uganda have revealed neither clinical nor subclinical infections of man by the agent (Kirya, G.B. personal communication, 1972). In Nigeria, on the other hand, a different situation exists. Between 1966 and 1974, 10 strains of a virus

indistinguishable by complement fixation (CF) test from the prototype Orungo virus strains have been isolated from man and mosquitoes in different parts of Nigeria (University of Ibadan Arbovirus Research Project, Annual Report, 1972).

In 1972, three outbreaks of a human disease characterised by nausea, vomitting, myalgia, headache, and fever of 3-7 days duration were reported from the Jos area of the Benue Plateau State of Nigeria, Fabiyi et al. (1975). Although no virus was isolated from samples collected during these outbreaks, over 60% of those affected reacted with significantly high CF antibody titres to Orungo virus only, thus demonstrating recent infection by the virus. Two deaths were reported during the outbreaks, however, the relationship of these deaths to the reported outbreak is not clear. Monath and co-workers, (1972) isolated three strains of Orungo virus from blood of febrile patients collected in the Anambra State during an epidemic of a human disease with signs similar to those of the Jos epidemic. Antibodies to Orungo virus were detected in the sera of other patients collected at the time of this epidemic.

Several reports of suspected outbreaks of yellow fever were investigated in 1973 and 1974 at Mabudi area a new settlement located some 300 km. from Jos at the base of the Plateau. Results of these investigations showed that Orungo virus was also active

at or about the same time as the yellow fever outbreak, Tomori et al. (1976). Similarly, during investigations into a dengue-like outbreak in humans at Abeokuta, Orungo virus was isolated from a febrile 13 year old girl, Fagbami et al. (1976). In Nigeria therefore, Orungo virus appears to constitute a public health hazard to the magnitude of its being considered a human infectious agent causing outbreaks or even epidemics. Moreover, the tendency of Orungo virus to appear along with other well known viral disease from which differential diagnosis is difficult makes Orungo virus worthy of a detailed study so as to throw more light on its role as an agent of human disease.

OBJECTIVES AND AIMS OF THIS STUDY

It is proposed to study Orungo virus using biochemical, biophysical and sero-immunologic methods with reference to:

1. Relationship to other viruses.
2. Antigenic analyses of Nigerian strains of Orungo virus and comparison with the original Uganda strain with a view to detecting reasons for the differences in biological behaviour of the virus strains in the two areas.
3. Determination of host range of infection using laboratory, domestic and wild animals.

4. Clinical disease symptoms associated with the virus and epidemiological studies to delimit the extent of infection in Nigeria.

It is hoped that these studies will lay a foundation for the detailed study of newly isolated viruses in Nigeria.

TABLE 1a

GROUPS, NAMES AND IBADAN PROTOTYPE NUMBERS OF VIRUSES ISOLATED IN NIGERIA 1964 - 1971

GROUP	TYPE	IBADAN PROTOTYPE
A	Chikungunya	H 35
	Igbo-Ora	H 10964+
	Sindbis	AN 47929
	Semliki Forest	AN 19809
B	Dakar bat	AN 8646
	Donguo 1	H 28328
	Donguo 2	H 11234
	Potiskum	AN 10069+
	Uganda S	AN 8829
	Wossolsbron	AN 31956
	West Nile	AN 4067
	Yellow fever	H 43913
	Zika	H 28444
Bunyamwera	Not typed	H 38684
Bwamba	Bwamba	H 75
	Pongola	AR 72850
Ganjam	Dugbe	AR 1792+
Nyando	Not typed	AR 15043
Phlebotomus fever	Arumowot	AN 14130

+ New viruses first isolated in Nigeria.

TABLE 1b

GROUPS, NAMES AND IBADAN PROTOTYPE NUMBERS OF VIRUSES ISOLATED IN NIGERIA 1964 - 1971

GROUP	TYPE	IBADAN PROTOTYPE
	Sud. AN 754-61	AN 10065
Piry	Chandipura	AN 9978
Simba	Ingwavuma	AN 28558
	Sabo	AN 9398+
	Sango	AN 5077+
	Sathuperi	AN 31273
	Shanonda	AN 5550+
	Shuni	AN 10107+
Ungrouped Mosquito borne	Orungo	H 11306
	Tataguine	H 9963
	Tete	AN 32897
	Rift Valley fever	AR 55171
Ungrouped Culicoides borne	Blue tongue	AR 27945
	E.H.D. related	AR 22619
	Abadina	AR 22388+
Ungrouped tick borne	Bhanja	AR 2709
	Congo	AN 7620
	Jos	AN 17854
	Thogoto	AR 2012
	Somone	AR 35018
	Nyamanini	AR 54516

+New viruses, first isolated in Nigeria.

TABLE 1c

GROUPS, NAMES AND IBADAN PROTOTYPE NUMBERS OF VIRUSES ISOLATED IN NIGERIA, 1964 - 1971

GROUP	TYPE	IBADAN PROTOTYPE
Rhabdoviruses	Mokola	AN 27377+
	Bovine ephemeral fever	AN 59689
	Rabies	AN 8574
	Kotonkan	AN 23380+
Others	African Horse sickness	AN 53177
	AN 2898	AN 2898+
	Herpes	H 21352
	NDV	AN 20433
	Poxviruses	AN 34325
	Coxsackie A 5	H 32075
	Coxsackie B 4	H 8874
	Echo type 11	H 9214
	Ib AN 28946	AN 28946+
	Eg AN 1398-61	AN 39652
	AN 54157	AN 54157+
	AN 17143	AN 17143+
	AN 33709	AN 33709+
	H 41795	H 41795+
	H 51378	H 51378+

+New viruses, first isolated in Nigeria.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Orungo Virus

For obvious reasons, very little is known about Orungo virus. The original strain, of the virus designated as UgMP 359 was an incidental isolation from a pool of 13 Anopheles funestus mosquitoes caught off human bait at Orungo, Teso District of Uganda, in 1959 during investigations of the O'nyong-nyong fever epidemic reported by Haddow et al. (1960). Not until seven years later in 1966, was another strain of the virus isolated this time in Nigeria from blood of a febrile child. Between 1966 and 1975, nine other strains of the virus were isolated from man and mosquitoes in different parts of Nigeria. Following the first isolation of Orungo virus in Nigeria in August 1966, from heparinised blood of a febrile child at the University College Hospital General Outpatients Clinic (UCHGOP), another strain was isolated also from heparinised blood of another febrile child in November of the same year at the UCHGOP. No further isolation was made until 1968, when the third isolate of Orungo was obtained from serum of a sick child (Annual Report, Virus Research Laboratory, Ibadan, 1969). The only Nigerian arthropod isolate of Orungo virus came from a pool of 17 engorged female Aedes dentatus mosquitoes collected at Du village near Jos, during follow-up studies on the vectors of

the 1969 yellow fever epidemic in that area (University of Ibadan Virus Research Laboratory, Annual Report, 1970). Four more isolates, all of human origin were isolated from blood of sick children at Ibadan (1) and East Central State (3), Monath et al. (op. cit.). In 1972, and 1973, one isolation each from Ilora and Abeokuta respectively was obtained from blood of febrile children. Isolations of Orungo virus have only been reported from Uganda, (1) Nigeria (10) and Central African Republic where one strain was isolated from a pool of Culex perfuscus mosquitoes (Robin, Y. and Sureau, P. personal communications, 1975).

2.2 Identification of the prototype strain from Uganda

Apart from the original Orungo virus strain, six other viruses were also isolated during the O'nyong nyong epidemic. Four were later identified as Bwamba virus strains, and one as Nyando virus, (Williams et al. op. cit.). The last, Tanga virus, was reported as a new virus by Woodall and Williams (1967). Initially, workers at the EAVRI reported relationships between Orungo and the other Bwamba virus strains using CF tests. However, further testing showed no relationships between Orungo and 27 other viruses including Bwamba isolated in Africa.

2.3 Properties of Orungo Virus

The agent killed one-day-old mice in six days at the original inoculation and in four days at subsequent passages. It passed through a Gradocol membrane of average pore diameter (APD) of 420 nm. Ether sensitivity was only 0.8 log difference between treated and control. The findings of the Uganda workers were confirmed by other workers at the Yale Arbovirus Research Unit (YARU) and Orungo virus was classified as a new ungrouped mosquito-borne virus (YARU, Annual Report, 1959).

Bordon et al. (1971) also confirmed the low sensitivity of Orungo virus to lipid solvents and based on this property and the lack of antigenic relationship to any major arbovirus serologic group A, B, and Bunyamwera, Orungo virus was placed along with 9 other viruses in the orbivirus taxonomic group. Although, there is no serological inter-relationship between Orungo virus and the other nine viruses of the group, Orungo virus has the typical electromicroscopic morphology of the orbivirus group (F. Murphy, personal communications, 1975).

2.4 Clinical symptoms associated with Orungo virus infection

Most of the isolations of Orungo virus were obtained from patients at the Outpatient Clinic, as such follow-up observations were difficult. The commonly described symptoms were fever (38°C-

40°C) of 3-7 days duration and generalised body pains. Familusi et al. (1972) described a case of a 2-year-old Nigerian girl, the blood of whom yielded one of the Nigerian strains of Orungo virus. The child presented with persistent fever and diarrhea of one month duration despite treatment with anti-malarials and antibiotics. On admission, temperature was 40°C with weakness of the lower extremities. The diarrhea finally subsided with treatment, but fever persisted for five days. Blood sample collected on admission yielded a strain of Orungo virus. The weakness of the lower extremities improved on recovery. It is difficult to ascribe all the other symptoms besides fever to Orungo virus.

Fabiyi et al. (op. cit.) described three outbreaks of an epidemic of human illness in Jos, Benue Plateau State of Nigeria. The disease was characterised by nausea, myalgia, headache and fever of 3-7 days duration. Although no virus was isolated, over 60% of cases were positive for Orungo CF antibodies only. Two deaths whose relationships to the outbreak was not clear were also reported in individuals with the described symptoms at the time of the epidemic.

Further serological evidence of infection by Orungo virus with a detailed description of clinical symptoms, was encountered in Ibadan (Ogunlesi, unpublished data). The illness was a short-term

febrile type presenting with conjuctivitis, muscle weakness, papular rash and skin tenderness. At the present moment, there are no pathognomonic symptoms associated with Orungo virus infection.

2.5 Antigenic variations between Orungo virus strains

In preliminary studies, slight antigenic differences have been observed between Nigerian strains and the original strain of Orungo virus from Uganda (Moore, Annual Report, 1969).

CHAPTER 3

MATERIALS AND METHODS

3.1 Isolation and identification of virus strains

Human blood specimens were obtained by finger prick drawn into heparinized cappilary tubes. Four or five tubes were filled from each patient, closed at one end with cricocaps and placed together in a numbered 12 x 17 mm tube. Whole blood, serum or both were diluted 1:4 with a diluent comprising 0.02 M phosphate buffered physiological saline (pH 7.2) to which was added 0.75% bovine plasma albumin (Armour fraction V) supplemented with 100 units/ml of penicillin and 100 micrograms per ml of streptomycin (BAPS), and inoculated IC into 2-3 day old baby mice. Blood specimens, collected from adults or during epidemiological investigations in and out of Ibadan were obtained by venepuncture. Sera were separated and kept in liquid nitrogen until arrival in Ibadan. Blood and organ collections from wild and domestic animals were also tested for Orungo virus isolation.

Mosquitoes caught by human bait or light trap as described by Lee (1969) were pooled by species and stage of feeding cycle in numbers usually not exceeding 15. They were then processed by standard techniques utilising IC inoculation of baby mice.

Presumptive serological identification was attempted on early mouse brain passages using stock mouse ascitic fluids and crude

antigens diluted in veronal buffer in CF tests. Definitive identification was made after the homologous immune MAF, and sucrose-acetone extracted antigen were available. The results of CF tests were confirmed by neutralization tests performed in 2-4 day-old suckling mice by IC inoculation.

Between 1966 and 1973, nine strains of Orungo virus were isolated in Nigeria. Eight of these were from human blood and the last from a pool of Aedes dentatus mosquitoes. These Nigerian strains, and two others, the prototype strain MP 359 from Anopheles funestus mosquitoes in Orungo, Uganda, and strain AR B 2078 from Culex perfuscus mosquitoes in N'dele, Central African Republic, are listed in Table 2, by location, source and date of collection. One other strain, M60974 originally reported as a strain of Orungo virus, by Monath et al. (op. cit.) was found during the course of these studies to be Tataguine virus.

All the strains used in these studies, had undergone varying numbers of intracerebral (IC) passage in suckling 2-3 day white Swiss albino mice. At the early passages, virus titers of most of the strains were low. To effect higher virus titers, mouse brain antigens at the lowest passage available were rapidly and successively passaged IC in mice. Briefly, ten-fold serial dilutions of infected mouse brains were made in BAPS. Each dilution was inoculated into a litter containing 6 suckling mice. Brains from sick mice at the

TABLE 2

ORUNGO VIRUS ISOLATES FROM NIGERIA AND OTHER PARTS OF THE WORLD, 1959 - 1973

ISOLATES	LOCATION	SOURCE	DATE OF SAMPLE COLLECTION
MP. 359	ORUNGO, UGANDA	Anopheles funestus	September 1959
H. 11306	IBADAN, NIGERIA	Human $2\frac{1}{2}$, M**	August 1966
H. 13019	IBADAN, NIGERIA	Human 4, M	November 1966
H. 30115	IBADAN, NIGERIA	Human 2, F	August 1968
AR.B. 2078*	N'DELE, CENTRAL AFRICAN REPUBLIC	Culex perfuscus	July 1969
AR. 52302	JOS, NIGERIA	Aedes dentatus	August 1970
H. 54760*	IBADAN, NIGERIA	Human 15, M	December 1970
H. 60784	ENUGU-EZIKE, NIGERIA	Human $1\frac{2}{12}$, M	August 1971
H. 60818	ETTEH, NIGERIA	Human 13, M	August 1971
H. 68367	ILORA, NIGERIA	Human 6, F	June 1972
H. 76247	ABEOKUTA, NIGERIA	Human 14, F	June 1973
H. 60974***	ABAKALIKI, NIGERIA	Human $1\frac{1}{2}$, F	August 1971

*Orungo virus strain not used in these studies.

**Age in years, and sex (human only).

***Tataguine virus originally identified as Orungo virus.

highest dilution were further diluted and inoculated into new litters of mice starting a dilution a step below that which yielded the sick mice. That is if the last dilution yielding sick mice was a $10^{-3.0}$ dilution, mouse brain from this dilution was serially diluted and inoculated into new baby mice starting from a $10^{-2.0}$ dilution. This was repeated until no significant increase in virus titre was noticed with further passages. Virus pool was then prepared for each isolate for use in the different tests during the course of these studies. The pool was prepared as follows: 0.02 ml of a 10% infected mouse brain in BAPS was inoculated IC into each of 10 litters of suckling mice. When mice were either sick or moribund, the brains were aseptically removed. A 20% suspension of infected mouse brain was prepared in BAPS and 0.5 ml dispensed into ampoules. The ampoules were divided into two groups.

Ampoules in the first group were sealed, rapidly cooled in liquid nitrogen and stored wet frozen at -70°C until used. The brain suspensions in the second group were lyophilised in an Edward's Freeze Dryer model EF 03 and stored at different temperatures until used.

The virus titre of each virus strain was determined by inoculating 10 fold serially diluted suspension of each strain into suckling mice. The inoculated mice were observed for 14 days after which infectivity titer was determined by the method of Reed and Muench (1938) and expressed in dex (= log 10, Haldane, 1960).

3.2 Antigen preparation

Sucrose-acetone extraction of infected mouse brain antigen according to the methods of Clarke and Casals (1958) was the source of antigen used in this study. Litters of suckling mice were inoculated IC, each mouse receiving 0.02 ml of a 10^{-2} dilution of infected mouse brain suspension in BAPS. Mice were observed daily and harvested when most were sick or moribund, and held frozen at -70°C until antigen was prepared. Mouse brains were aseptically removed by suction applied through a 20 ml syringe with an 18 guage needle attachment. The brain harvest collected into a universal bottle, was then weighed and homogenised in 4X (V/W) of a 0.3% aqueous solution of sucrose. Homogenization was achieved by 3-4 minute sonication cycles with a sonifier, each cycle interspersed by 1 minute rest periods. The homogenized brain suspension was next added slowly and dropwise into a continuously agitated flask containing chilled acetone. Final acetone: homogenate proportion was twenty to one. The flask with the content was then vigorously shaken and left for a few minutes to allow particles to sediment. The acetone was then aspirated off, and another 20 volumes of fresh chilled acetone added, and left at 4°C for 1 hour. The acetone was again aspirated off and the precipitate dried using a vacuum pump for 45-60 minutes. All manipulations during extraction and drying were carried out in ice bath. The dried residue was broken

up and resuspended in a volume of physiologic saline twice the weight of the original brain harvest. The resuspended residue was allowed to stand overnight at 4°C. The resultant suspension was centrifuged in a PR 2 International centrifuge at 3,000 revolutions per minute (rpm) for 20 minutes with temperature setting at 4°C. The supernatant fluid, that is, the antigen, was transfered to Bijou bottles in 1 ml aliquots and stored at -70°C until used.

3.3 Preparation of immune ascitic fluids

Immune mouse ascitic fluid (MAF) was prepared to each strain of Orungo virus in 6-9 week old Swiss albino mice. Increasing level of immunity was achieved by inoculating mice with 0.2 ml amounts of one, two or four intra-peritoneal (IP) injections of inactivated or live virus preparations as shown in Table 3. Inactivated antigens were prepared by the addition of betaproprolactone (BPL) in distilled water to a 10% suspension of infected suckling mouse brain tissue in physiological saline held overnight at 4°C. Final BPL concentration was 0.05% (V/V). Live virus preparation was a 10% suspension of infected mouse brain in physiological saline. Both the inactivated and live virus suspensions were mixed with equal volume of Fruend's complete adjuvant (FCA) as described by Tikasingh, Spence & Downs (1966), before each injection. Sarcoma 180/TG cells were given in 0.2 ml amount by IP

TABLE 3

INOCULATION SCHEDULES OF ADULT MOUSE FOR THE PRODUCTION OF IMMUNE MOUSE ASCITIC FLUIDS (IMAFS) TO ORUNGO VIRUS STRAINS

Day	INOCULUM			Amount per mouse	Route
	1 SHOT	2 SHOTS	4 SHOTS		
1	Virus + BPL + FCA	Virus + BPL + FCA	Virus + BPL + FCA	0.2 ml	IP
7	Sarcoma-180/TG Cells	Sarcoma-180/TG Cells	Virus + BPL + FCA	0.2 ml	IP
14	- +++	Virus + FCA	Virus + FCA	0.2 ml	IP
21	-	-	Sarcoma-180/TG Cells	0.2 ml	IP
28	-	-	Virus + FCA	0.2 ml	IP

BPL = Beta-propiolactone.

FCA = Fruend's Complete adjuvant.

+++ = no inoculations.

inoculation as employed by Sartorelli, Fisher and Downs (1966). First tapping of mice was carried out when most mice were distended, between 10-12 days after the sarcoma 180 TG injection; thereafter tapping was done depending on state of distension of mice. Each tapping was lightly centrifuged to remove the cells and the supernatant fluid, containing the antibody was stored frozen at -70°C. Different tappings were tested in complement fixation (CF) tests against the homologous antigen, and pooled according to the level of CF titre.

3.4 Preparation of complement fixation (CF) test materials

3.4.1 Sheep redblood cells

Sheep red blod cells were used as CF indicator. The whole blood was collected in an anticoagulant, Alsever's solution, in a proportion of 8.5 ml of blood to 1.5 ml Alsever's solution. The blood was washed once in saline and twice in veronal buffered diluent (VBD). A 5% suspension of washed cells was made in VBD and kept at 4°C until used. Alsever's solution and VBD were prepared as shown below.

ALSEVER'S SOLUTION

Dextrose	20.5 g
Sodium Chloride	4.2 g
Citric acid	0.55 g

ALSEVER'S SOLUTION (contd.)

Sodium citrate	88.0 g
Distilled water q. s ad	1000 ml

Sterilized by autoclaving for 10 minutes at 10 lb. pressure.

VERONAL BUFFERED DILUGENT (VBD)

Dissolve 1 tablet of Oxoid CFT diluent tablet in 100 mls. of distilled water with warming.

Formula for Oxoid (1969) CF diluent tablet

Barbitone	0.575 g
Sodium chloride	8.500 g
Magnesium chloride	0.168 g
Calcium chloride	0.028 g
Barbitone soluble	0.185 g

All dissolved in 1 litre distilled water to give a pH of 7.2.

3.4.2 Hemolysin

Hemolysin, anti-sheep red blood cell rabbit serum was used to sensitize erythrocytes. The serum was titrated as shown below and used at 4 to 6 mean hemolytic doses (MHD).

First, three master dilutions 1:10, 1:100 and 1:1000 were made as follows:

1 volume of serum + 9 volumes of VBD = 1:10

1 volume of 1:10 serum + 9 volumes of VBD = 1:100

2 volumes of 1:100 serum + 18 volumes of VBD = 1:1000.

From the above the other hemolysin dilutions were made

Final hemolysin dilution	Volume of VBD	Volume of 1:10 hemolysin
1:10	0	2
1:50	4	1

Final hemolysin dilution	Volume of VBD	Volume of 1:100 hemolysin
1:100	0	2
1:200	1	1
1:400	3	1
1:800	7	1

Final hemolysin dilution	Volume of VBD	Volume of 1:1000 hemolysin
1:1000	0	2
1:1500	1	2
1:2000	2	2
1:2500	3	2
1:3000	2	1
1:4000	3	1
1:5000	4	1

For titration purposes, one volume of appropriate hemolysin dilution was added to one volume of 4% sheep red cells and incubated in water bath at 37°C for 15 minutes. The dilution series 1:10 to 1:800 was removed to stand at room temperature to observe agglutination. Agglutination

should not occur in 6 MHD of hemolysin and preferably not exceed 1:100 hemolysin dilution. To dilutions from 1:1000 to 1:5000 was added 1 volume of 1:10 complement dilution and 1 volume of diluent and incubated at 37°C for 15 minutes. The highest dilution showing complete hemolysin was taken as 1 MHD.

3.4.3 Complement

Fresh or reconstituted lyophilized guinea pig serum was the source of complement. The complement was titrated as follows: Complement diluted to 1:30 was distributed as shown below into 8 master tubes.

Reagent (ml)	Tube Number							
	1	2	3	4	5	6	7	8
VBD	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2
1:30, Complement (C1)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8

Corresponding titration tubes were set up to which 0.2 ml of complement from each master tube was transfered. Other reagents were added to the tubes as shown below:

Reagent (ml)	Tube Number							
	1	2	3	4	5	6	7	8
Complement (C!)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
VBD	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
4% sensitized sheep cells	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final volume of 1:30 C! in tube	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08

The titration tubes were incubated in water bath at 37°C for 30 minutes after which the titration was read. The tube showing complete hemolysis was the end point which contained 1 unit of complement.

Formula for calculating the correct dilution required to give two units of complement is as follows:

$$\frac{\text{Reciprocal of original dilutions of complement}}{\text{Twice the amount of C!. in the tube}} \times 0.1$$

For example, if complete hemolysis was in Tube 5 the C! dilution needed is $\frac{30}{.05 \times 2} \times 0.1 = 1:30$

3.5 Materials for hemagglutination (HA) and hemagglutination-inhibition (HI) tests.

3.5.1 Red blood cells

Goose, goat, sheep, chicken (rooster), Patas and Rhesus monkey, and human erythrocytes were tested as HA and HI indicators as used by Banerjee (1965). 17 mls of blood was collected into 3 ml of acid-citrate-dextrose (ACD) anti-coagulant. Goose and chicken were bled from the wing vein, and the rest from the jugular vein. The erythrocytes were washed 4 times with ice cold dextrose gelatin-veronal (DGV) solution, using 2.5 volumes of DGV for 1 volume of whole blood for the first wash, and 3 volumes DGV for subsequent washes. At each washing, the suspension was centrifuged at 1,500 rpm in the refrigerated PR2 international centrifuge for 15 minutes; and supernatant discarded. All manipulations were carried out in sterile glassware and with aseptic precautions. After the 4th washing the red blood cells were suspended in DGV and stored at 4°C as a 10% suspension.

3.5.2 Treatment of immune mouse ascitic fluid for HAI tests

3.5.2.1 Kaolin absorption

A slurry of 25 percent kaolin was prepared by adding 25 gram of the acid-washed kaolin powder to 100 ml of borate saline solution, pH 9.0, with constant mechanical stirring for maximal

suspension. To 0.4 ml of ascitic fluid were added 1.6 ml of borate saline pH 9.0 and 2.0 ml of the kaolin mixture. The mixture was shaken vigorously at 5 minutes interval for 20 minutes at room temperature and centrifuged at 1,500 rpm for 30 minutes. The supernatant fluid represented a 1 in 10 dilution of the original ascitic fluid.

3.5.2.2 Acetone extraction

0.2 ml of immune ascitic fluid was diluted 10-fold with 0.85 per cent saline. To the tube of diluted immune fluid cooled in an ice water bath was added 2½ ml of chilled acetone. Extraction was allowed to take place for 5 minutes with intermittent shaking of the solution. The tube was centrifuged at 2,500 rpm for 5 minutes at 4°C. The supernatant fluid was carefully aspirated from the tube and the sediment was resuspended by vigorously shaking with another 2½ ml chilled acetone. Centrifugation was repeated and after aspiration of the supernatant fluid, the sediment was dried under vacuum at room temperature for about 30-60 minutes. The dried sediment was resuspended in 1.8 ml borate saline solution pH 9.0 to make a 1:10 dilution of the original test immune fluid. The suspension was allowed to stand overnight in the refrigerator.

3.5.2.3 Agglutinin adsorption

To the kaolin - or acetone - treated 1:10 immune fluid dilution was added 0.05 ml of packed red cells. The treated immune fluid was

held in an ice bath to prevent hemolysin. Adsorption occured within 20 minutes with occasional shaking after which the suspension was centrifuged at 1,500 rpm for 10 minutes at 4°C. The resulting supernatant fluid was then ready for testing.

3.5.3 Reagents for HA and HI tests

All reagents were prepared in glass distilled or demineralized water.

STOCK SOLUTIONS

1.5M sodium chloride (10 x 0.9% NaCl)

NaCl	87.675 g
Distilled water q.s. ad	1000 ml.

0.5M Boric acid

H_3BO_3	30.92 g
Distilled water q.s. ad	1000 ml.

First dissolve in 700 ml hot distilled water, cooled and made up to 1000 ml.

1.0M Sodium hydroxide

NaOH	40.0 g
Distilled water q.s. ad	1000 ml.

2.0M Disodium hydrogen phosphate

Na_2HPO_4	283.96 g
Distilled water q.s. ad	1000 ml.

2.0M Sodium dihydrogen phosphate

NaH_2PO_4	276.02 g

Distilled water q.s. ad 1000 ml.

B. Borate-saline pH 9.0 (0.05M borate - 0.12M NaCl)

1.5 M NaCl	80 mls
0.5 M H_3BO_3	100 mls
1.0 M NaOH	24 mls

Distilled water q.s. ad 1000 mls.

B_1. Borate-saline pH 9.3

Same as for B but used 31 mls of 1.0M NaOH.

C. Acid-Citrate-Dextrose (ACD)

Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)		11.26 g
Citric acid ($H_3C_6H_5O_7.H_2O$)		4.0 g
Dextrose	11	11.0 g
Distilled water q.s. ad		500 mls

Sterilized by autoclaving for 10 minutes at 10 lb pressure.

D. Dextrose-gelatin-veronal (DGV)

Veronal (Barbital)	0.58 g
Gelatin	0.60 g
Sodium veronal (Sodium barbital)	0.38 g
$CaCl_2$ anhydrous	0.02 g
$MgSO_4 . 7H_2O$	0.12 g.
NaCl	8.5 g
Dextrose	10.0 g

First veronal and gelatin were dissolved in 250 mls of hot water, before adding the other reagents and making up to 1000 mls with distilled water.

E. Bovalbumin-borate saline (BABS)

Antigen-serum diluent 0.4% bovine albumin in borate saline pH 9.0

i. 4% Bovalbumin (Fraction V).

Bovalbumin	4 g
Borate saline solution pH 9.0 q.s. ad	100 ml

ii. Antigen-serum diluent: 0.4% BABS

4% Bovalbumin pH 9.0	100 mls
Borate saline solution pH 9.0	900 mls

G. Virus adjusting diluent (VAD)

Solution A: 0.15M NaCl - 0.02M Na_2HPO_4

1.5M NaCl	100 ml
2.0M Na_2HPO_4	100 ml
Distilled water	8000 ml.

Solution B: 0.15M NaCl - 0.2M NaH_2PO_4

1.5M NaCl	100 ml
2.0M NaH_2PO_4	100 ml
Distilled water	800 ml.

Table of pH values

Final pH	Solution A 0.15M NaCl - 0.2M Na_2HPO_4	Solution B 0.15M NaCl - 0.2M NaH_2PO_4
5.75	3.0 ml	97.0 ml
6.0	12.5	87.5
6.2	22.0	78.0
6.4	32.0	68.0
6.6	45.0	55.0
6.8	55.0	45.0
7.0	64.0	36.0
7.2	72.0	28.0
7.4	79.0	21.0

3.6 Preparation of materials for gel diffusion

The agar-gel precipitation test of Ouchterlony (1958) and the template thin-layer gel diffusion method as described by Auernheimer and Atchley (1962) were used. The latter is a micro-technique for conducting the Ouchterlony test.

For the routine Ouchterlony test, microscope slides throughly cleaned in distilled water were left in acetone until used. The clean and dry slides were coated with a 0.1% molten Difco Noble agar in distilled water, and left to dry at room temperature overnight. Three milliliters of 1% agar in distilled water containing a few drops of 1% of sodium azide as a preservative was layered on each slide and allowed to gel at room temperature. The slides were stored in a humid chamber at 4°C until used, usually within two to three days. Desired patterns were cut into the agar with a Gelman gel punch (Clinical electrophoresis, 1970) and extracted by suction. Wells were filled with antigen or mouse ascitic fluids and diffusion allowed to take place at room for 24-48 hours in a humidified chamber. Slides were stained with the following dye after precipitin lines had developed:

Thiazine red	0.1 g
1% acetic acid	100.0 ml.

The apparatus for the Auernheimer and Atchley modification included diffusion chamber, templates and gel matrix forms. The

diffusion chamber was a combined chamber and slide rack fabricated from acrylic plastic sheet as described by Auerheimer and Atchley (op. cit.). It was used for all steps of slide preparation, diffusion, elution, staining and drying.

Templates were pieces of plastic 1" x 1¼" x $\frac{1}{16}$" cut from acrylic plastic sheet. Holes, usually $\frac{1}{8}$" diameter were drilled in the plastic pieces in the desired patterns. Well patterns used in this study are shown in Figure 19.

Gel matrix forms were strips of vinyl plastic tapes $\frac{1}{8}$" x 1" placed each at end of the template. Microscope slides were factory precleaned slides left in acetone until used. A 0.3% Difco Noble agar in distilled water was employed to coat the slides which were left in a near vertical position to dry at room temperature. Dry precoated slides were then placed on a support to raise them off of the bench top. A gel matrix form was placed on the slides, tape down. Agarose (0.5%) at 60-90°C was introduced into the capillary space between the slides and the gel with a Pasteur pipette fitted with a rubber bulb. After storing in the moist chamber until the agarose was well gelled (usually after 1 hour), the gel form was removed by sliding it off to one side. In place of the gel form a template on the desired well pattern was slid on, starting at one edge in order to prevent air bubbles from being trapped. The wells were filled with the appropriate solution of

antigen or mouse ascitic fluid using a capillary tube. The top of the capillary was placed close to the bottom of the well and solution was allowed to gently flow until the well was full, avoiding air bubbles. Filled slides were then placed in the moisture saturated chamber and kept at room temperature for 24-48 hours. At the end of the diffusion period, the template was removed from the slide by flowing a gentle stream of tap water over the assembly, while the template was carefully slid off to one side. Before staining the slides, unreacted antigen or mouse ascitic fluid were eluted by three to four changes of buffer solution over a 4 hour period. Slides were stained with 0.1% thiazine Red in 1% acetic acid. The buffer for preparing the 0.5% agarose and for elution was made up of the following:

Sodium dihydrogen phosphate ($NaH_2PO_4 . H_2O$)	0.15 g
Disodium hydrogen phosphate Na_2HPO_4	1.25 g
Sodium Chloride NaCl	8.5 g
Sodium azide NaN_3 (preservative)	0.2 g
Water q.s. ad	1000 ml.

Final pH is 7.5.

3.7 Tissue culture

Three established cell lines were used during the course of these studies. These were African green monkey, Cercopithecus aethiops kidney (Vero) cells, Syrian or Golden Hamster, Mesocricetus

auratus kidney (BHK-21) cells, and Aedes albopictus (A. alb.) cells. The vero and BHK-21 cells were obtained from the American Type Culture Collection and the A. alb. was the Singh. Spring 1970 subline maintained by Dr. S. Buckley at the Yale Arbovirus Research Unit, New Haven, Connecticut, U.S.A.

Stock Vero cell cultures were carried in Roux bottles with a growth medium consisting of 90% Eagles Minimal Essential Medium (MEM) with Hank's balanced salt solution (HBSS) and 10% Fetal calf serum (FCS). Cultures were transfered weekly by a 1:4 split. Two-ounce flint glass prescription bottles were seeded with cell suspension from stock culture containing 150,000 cells/ml, each bottle receiving 5 ml. These cultures were used for virus assay 3-4 days after seeding tubes with 1 ml of cell suspension each.

The stable BHK-21 cell line was grown in French square bottles in a medium consisting of 90% MEM made up with HBSS and 10% FCS, these cultures were transferred twice weekly by a 1:4 split. Tube cultures for virus studies were usually prepared by seeding 50,000 cells/ml in an outgrowth medium of MEM: HBSS: FCS at 80:10:10 ratio.

A. alb. stock cells were maintained in 2-ounce flint glass prescription bottles with 5 ml of Mitsuhashi-Maramorosch (M-M) medium. Each week, the M-M medium was drawn off and replaced with an equal volume of fresh medium. For virus assay, each stock bottle

was transfered by a 1:8 split ratio. Medium was poured off the bottle and cells detached from glass wall with a "rubber policeman". Three ml of medium was added and cell suspension pipetted up and down 10-20 times to break cell clumps. To the dispersed cells was added enough medium to make up 8 new stock bottles containing 5 ml of cell suspension each. The bottles were incubated for 3 days at room temperature by which time the cell monolayer was suitable for virus inoculation.

For virus inoculation, the fluid medium was removed from the confluent monolayer cultures and 0.1 ml (tube) or 0.2ml (2 ounce flint glass bottles) of virus dilution was adsorbed for 1 hour at 37°C or room temperature. This was followed by the addition of MEM with 2% FCS for Vero or BHK-21 cells, and the M-M medium for A. alb. cells.

For plaque formation assays, freshly prepared nutrient agar overlay medium was added to confluent monolayer culture after virus adsorption. Two procedures were employed for overlaying infected monolayer cultures. The single overlay medium in which neutral red dye was incorporated and the double overlay consisting of an initial medium without neutral red and a secondary medium with neutral red.

Each 2 ounce bottle received 5 ml of the single overlay or 3 ml of the initial and 2 mls of the secondary over ay medium

when the double overlay medium was used. The secondary overlay was added 3 days post viral infection.

3.8 Preparation of Mitsuhashi-Maramorasch (1964) medium and Rinaldini's (1954) solution for A. alb. cell line

3.8.1 Mitsuhashi-Maramorosch medium

Two solutions A and B were prepared in distilled demineralised water and kept for long periods at 4°C.

SOLUTION A

$NaH_2PO_4.H_2O$	2.50 g
$MgCl_2.6H_2O$	1.25 g
KCl	2.50 g
$CaCl_2.H_2O$	2.50 g
NaCl	87.5 g.

Demineralised distilled water q.s. ad 1000 ml.

SOLUTION B

$NaHCO_3$	1.50 g

Demineralised distilled water q.s. ad 1000 ml.

BASIC M-M (SINGLE STRENGTH) MEDIUM

Into a 2 litre flask, add

Dextrose	5.0 g
Lactalbumin hydrolysate	8.15 g
Yeastolate	6.25 g.
Demineralised distilled water 800 ml.	

Dissolve and hold overnight at 4°C. Next heat to approximately 80°C in a water bath and allow to cool. Then add

Solution A	100 ml
Solution B	100 ml.

Sterilize by filtration through a Seitz-type pad through which 50 ml of isotonic saline had been passed prior to filtration of basic medium.

To 800 ml of the filtered basic medium is added -

Heat inactivated fetal calf serum	200 ml
Antibiotic solution	10 ml.

The antibiotic solution contains 10,000 units of penicillin/ml and 10,000 micrograms of streptomycin/ml.

3.8.2 Rinaldini's salt solution

This is used for rinsing A. alb. culture following virus adsorption -

NaCl	0.8 g
KCl	0.02 g
$NaH_2PO_4.H_2O$	0.005 g
D-glucose	0.1 g
$NaHCO_3$	0.1 g

Rinaldinis salt solution (contd.)

Sodium citrate	0.0676 g
Antibiotic solution	10.0 ml.
(to give 100 units/ml of penicillin and 100 ugm/ml of streptomycin)	
Distilled water q.s. ad	100 ml.

Sterilised by filtration before adding antibiotic solution.

3.9 Preparation of overlay medium for plaque assay of Orungo virus

Agar overlay medium Medium M 199 (DOUBLE STRENGTH)

10 x M199 with Earle's base	200 ml
Inactivated fetal calf serum	20 ml
Antibiotic solution (to give 100 units/ml of penicillin and 100 ugm/ml of streptomycin)	4 ml
Distilled water q.s. ad	1000 ml.

2% agar solution

Difco Noble Agar	20 g
Distilled water	1000 ml.

Autoclave at 10 lb pressure for 15 minutes.

1% DEAE-Dextran

DEAE-dextran	10 gm
Distilled water	1000 ml

Autoclave at 10 lb pressure for 15 minutes.

Single overlay medium

To a 500 ml graduated cylinder add -

$NaHCO_3$	30 ml
M199 (2X) medium q.s. ad	250 ml
Neutral Red (1:30)	3.5 ml
M199 (2X) medium q.s. ad	500 ml.

To another 500 ml graduated cylinder add -

1% DEAE-dextran	30 ml
2% Noble agar	500 ml.

Mix content of both cylinders and in a flask and hold in a 44°C water bath while in use.

Double overlay medium

Initial overlay

To a 500 ml graduated cylinder add -

7.5% $NaHCO_3$	30 ml
M199 (2X) medium q.s. ad	500 ml.

To another 500 ml graduated cylinder add -

1% DEAE-dextran	30 ml
2% Noble agar q.s. ad	500 ml.

Mix contents of the 2 cylinders in a flask and hold in a 44°C water bath while in use.

Secondary overlay (added 3rd day after virus inoculation)

To a 500 ml graduated cylinder add -

7.5% $NaHCO_3$	30 ml
M199 (2X) medium q.s. ad	250 ml
1% Neutral red	4 ml
M199 (2X) medium q.s. ad	500 ml

To another 500 ml graduated cylinder add -

1% DEAE-dextran	30 ml
2% Noble agar q.s. ad	500 ml.

Mix contents of the 2 cylinders in a flask and hold in a 44°C water bath while in use.

3.10 Susceptibility studies with Orungo virus

Six experimental animals were used in the host range susceptibility studies with Orungo virus. These were white Swiss albino mice, hamsters (Mesocricetus auratus), white rabbit (Oryctolagus cuniculus), lambs, domestic sparrow (Passer domesticus) and domestic chickens (Gallus domesticus).

The white Swiss albino mice were maintained in the Virus Research Laboratory at Ibadan. They were used at different age groups ranging from one day old to 3-week old. Hamsters were obtained from the colony maintained at the Vector Borne Disease Division (VBDD) of the Centre for Disease Control at Fort Collins,

Colorado in the U.S.A. They were used at 2 day or 3-week of age. Adult rabbits (8-10 weeks) were obtained from a commercial rabbit farm in Ibadan. They were observed in the laboratory for ten days and certified free of any obvious disease prior to commencement of experiment. The adult sparrows were wild caught birds maintained at VBDD in Fort Collins, U.S.A. Baby chicks were obtained as day-old chicks from a commercial firm. Lambs were cross of local ewes and rams maintained in our laboratory.

CHAPTER 4

EXPERIMENTAL AND RESULTS

4.1 Filtration of Orungo virus strains through "Millipore" membranes

As a pre-requisite for further detailed studies, it was necessary to determine if the agent satisfies one of the criteria of viruses, that is, filterability. The technique described by Casals (1968) was used. Briefly a 10^{-2} suspension of infected newborn mouse brain tissue was prepared in BAPS solution. Between 50–60 ml of the freshly prepared virus suspension was centrifuged at 12,000 - 15, 000 rpm for 30 minutes. The supernatant fluid was then filtered by pressure (20 psi) successively through filters of average pore diameters (APD) 450, 300, 220 and 100 nanometers (nm). At each stage, about 5 ml was reserved for infectivity titration. The original crude suspension, the supernatant fluid and each successive filtrate were titrated by IC inoculation of 2–4 day old mice, six mice being used for each dilution. Infectivity titers were expressed in dex/0.02 ml (Haldane, op. cit.).

The results of infectivity titrations of four strains of Orungo virus are shown in Table 4. No significant loss in infectivity was detected on filtration through the APD 450-220 nm. However on filtration through the membrane of APD 100 nm greater than 1.5 dex of virus was lost. A loss of 1.5 dex or more in

TABLE 4

INFECTIVITY OF ORUNGO VIRUS STRAINS AFTER FILTRATION THROUGH "MILLIPORE" MEMBRANES.

VIRUS STRAIN	Infectivity of unfiltered suspension	LD_{50} TITER OF FILTRATE APD OF MEMBRANES IN NANOMETERS				Estimated Diameter of Particle (EDP)* in nanometers
		450	300	220	100	
M13019	4.3**	3.7	3.4	3.3	1.2	100 EDP 220
AR52302	5.4	5.0	5.0	4.8	2.6	100 EDP 220
H60818	3.2	3.0	3.2	2.3	0.6	100 EDP 220
UgMP 359	4.5	3.9	3.8	3.8	2.0	100 EDP 220

*Based on the average pore diameter (APD) of the two consecutive filters between which dex 1.5 or more of virus was removed.

**Expressed in dex/0.02 ml.

EPD = Estimated Particle Diameter.

infectivity titre was considered as significant. Orungo virus has an estimated particle diameter (EPD) of less than 220 nm but greater than 100 nm.

It must be noted that properties other than size may affect the filtrability of viruses and the commercially available membrane represent only a limited range of MPD, so the EPD obtained for Orungo virus is only a rough estimate rather than an accurate size determination.

Black (1958) arrived at a figure of 0.64 as the relation between particle size as determined by electron microscopic observation and the filtration technique. Orungo virus particles therefore should have a size between 64 nm and 140 nm. For an accurate determination of Orungo virus particle size, as well as the morphological and morphogenetic characteristics, virus grown in tissues culture and mouse brain was examined by electron microscopy.

4.2 Electron microscopy of Orungo virus in tissue culture and mouse brain

The Ib H 13019 strain of Orungo virus was used in these studies. It had previously been passaged eight times intracerabmally in suckling mice. Stock virus titer was 7.0 dex/0.02 ml.

BHK-21 cell cultures were infected with 0.1 ml of a 10^{-2} dilution of Orungo virus. Cells were harvested at the earliest signs of cytopathology (CPE), usually at 48 hours for thin-section electron microscopy, and at a later stage (60-72 hours) for negative contrast microscopy. Newborn baby mice were each inoculated IC with 0.02 ml of 10^{-2} dilution of virus and harvested when sick or moribund.

For thin section microscopy, cell cultures were scraped from bottle and centrifuged at 1000 rpm for 10 minutes, resulting pellets were fixed for 2 hours at 4°C in 2.5% glutaraldehyde. Mouse brain tissue was cut into 1 mm^3 blocks and treated in the same manner as cell culture pellets. Specimens were post fixed in 1% osmium tetroxide for 30 minutes, dehydrated in a standard ethanol series and embedded in an Araldite-epon mixture, (Mollenhauer, 1964). Sections were stained with uranyl acetate and lead citrate.

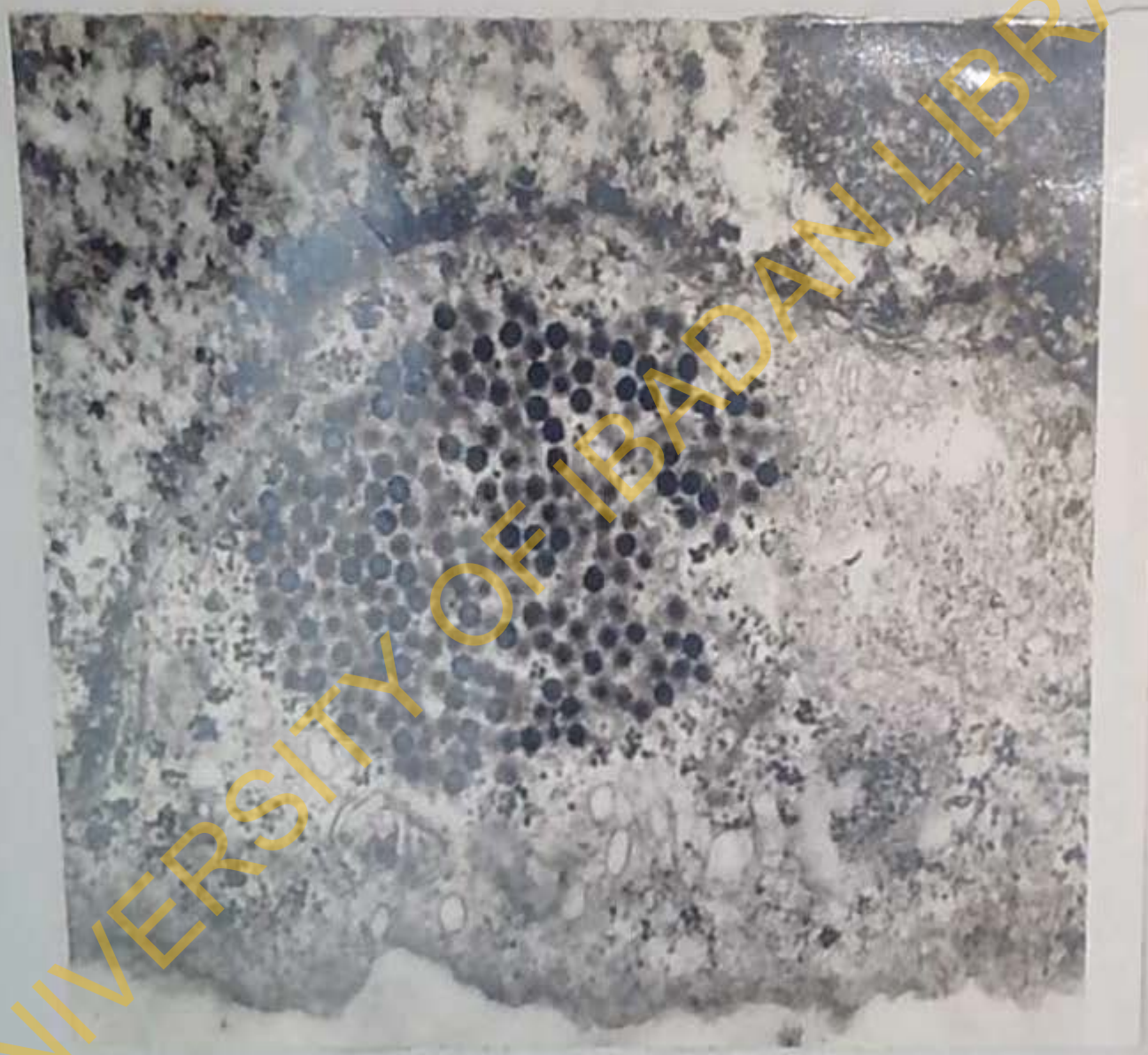
For negative contrast microscopy, several methods of virus growth and preparation were examined in order to obtain optimal resolution of particle surface structure. Virus was grown in 2, 5 or 10% fetal calf serum, and harvested at 12, 24, 48 and 72 hours. Cell disruption was carried out by freeze-thawing, mechanical disruption and trypsin treatment. In addition, partially purified virus was examined. Purification was carried out by previously

described methods, Martin et al., 1975; and Martin and Zweerink (1972). Briefly, medium was poured off infected cells, and replaced with one-tenth volume of water. Cells were then scraped and lysed in a homogenizer. The lysed cells were thereafter treated twice with equal amounts of fluoro carbon, Genetron; on each occasion the aqueous phase was collected. The pooled aqueous phase was layered onto a 30% glycerol - 60% potassium tartarate gradient, and centrifuged at 40,000 rpm for 19 hours in a SW 41 Spinco rotor. Two bands resulted, an upper thin precise band containing the virus particles, and a lower fuzzy band containing cellular debris. Specimens from these bands were prepared for electron microscopy by the pseudo replica technique of Sharp (1960) as fully described by Smith (1960). In this method, 2% potassium phosphotungstate was used as the negative contrast medium.

In thin section electron microscopy, of infected BHK-21 cells, Orungo virus particles were found located in the cytoplasm of infected cells (Plate 1). Most often, these masses were associated with granular matrices of varying densities and masses of filamentous structures (Plate 2). The variety of these aggregations were exceptional, in particular was the arrangement of virus particles around the periphery of mitochondira in some infected cells (Plate 3).

Few virus particles were observed in the process of budding through cell membranes, either through intracytoplasmic organelle

Plate 1

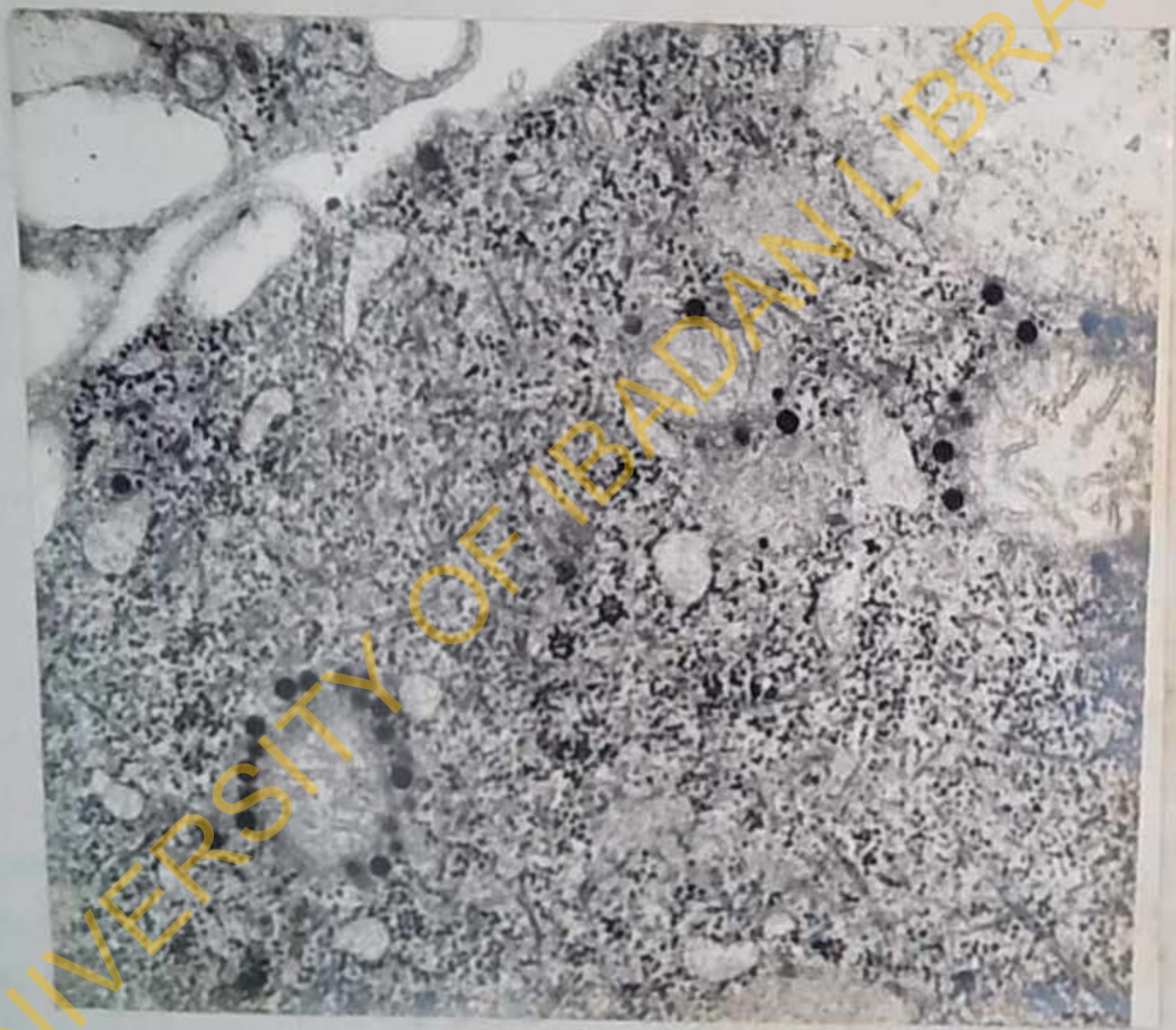


Orungo virus in BHK-21 cells, with virus particles lying free in massed array in the cytoplasm. X48,000.

Plate 2

Orungo virus in BHK-21 cells showing complex array of virus particles, several densities of viral matrix material and associated filaments in infected cells. X32,000.

Plate 3

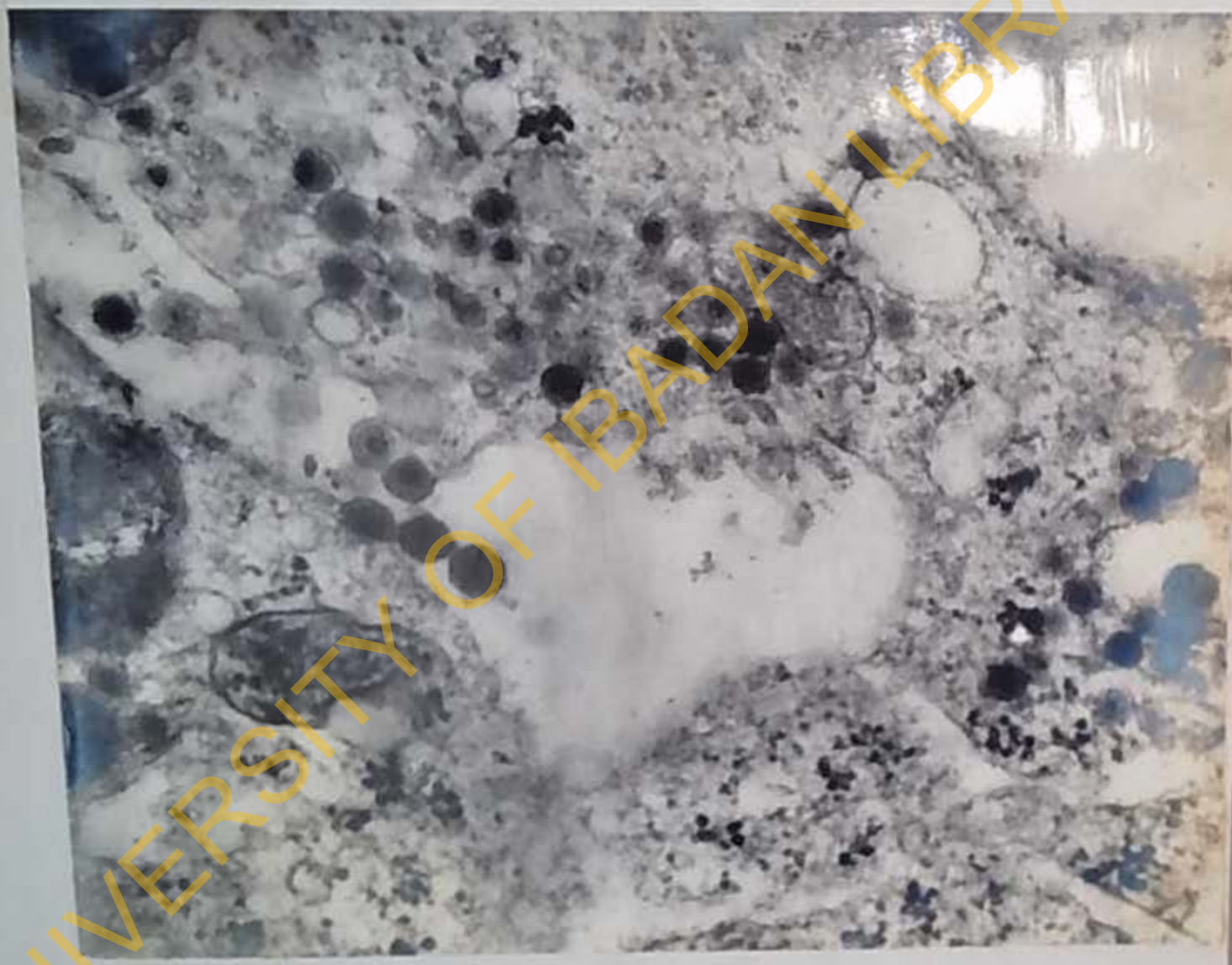


Orungo virus particles arranged at periphery of mitochondria in an infected BHK-21 cell. X38,000.

membranes yielding virus particles free in vacuoles or through plasma membranes yielding virus particles free in extracellular space before cell lysis (Plate 4). These particles maintained their "pseudo-envelope" as the membrane through which they budded remain adherent. The major means of virus release was via cell lysis and most released virus particles were not enveloped. In thin section, Orungo virus particles consisted of an electron dense core and a less dense outer shell, the capsid. The mean diameter of 105 Orungo virus particles was 63 nm with a range of 52-72 nm. Core diameter was 34 nm (range 29 nm - 41 nm). The distribution of Orungo virus particle diameter is shown in Fig. 1.

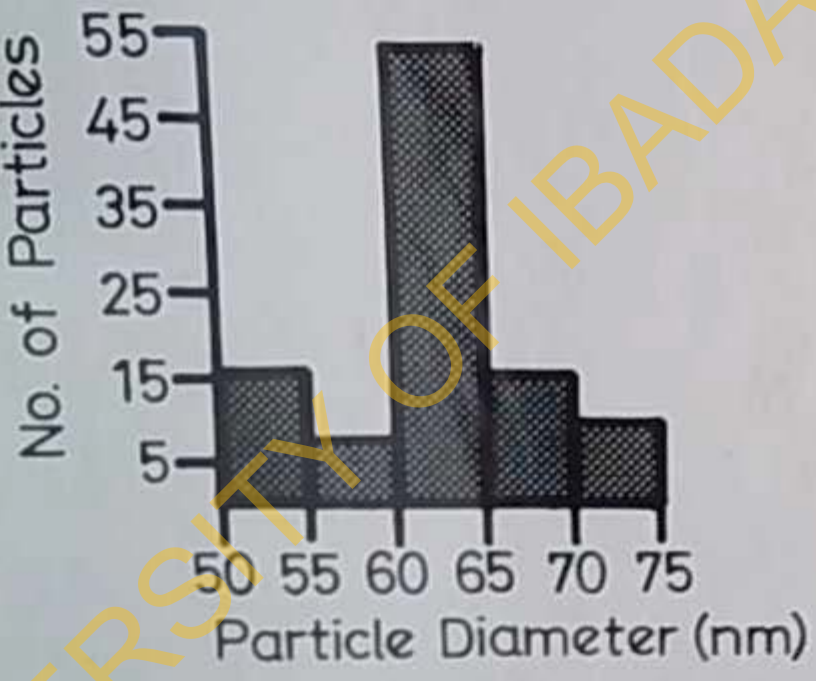
Negative contrast electron microscopy: Little surface structure was revealed by any of the treatments employed, including the partial purification by centrifugation on glycerol-tartarate gradients. In most preparations particle surfaces were fuzzy because of the presence of an indistinct layer overlying the isometric capsids. In an attempt to resolve this partially purified virus preparations were mixed with an equal volume of a 0.05% Difco 250 typsin in 0.01M sodium phosphate-buffered saline, pH 2.7 and incubated at 37°C for 1 hour. Samples were taken at 15 minute interval for electron microscopy. There was deep penetration of particles by the negative contrast sodium, resulting in very thin electron-transparent layer basal to surface capsomeres,

Plate 4



Orungo virus particles budding through intracytoplasmic membranes yielding "pseudo enveloped" forms. X111,100.

Fig. 1



Distribution of Orungo virus particle diameters.

but this did not help resolve capsid structure (Plate 5).

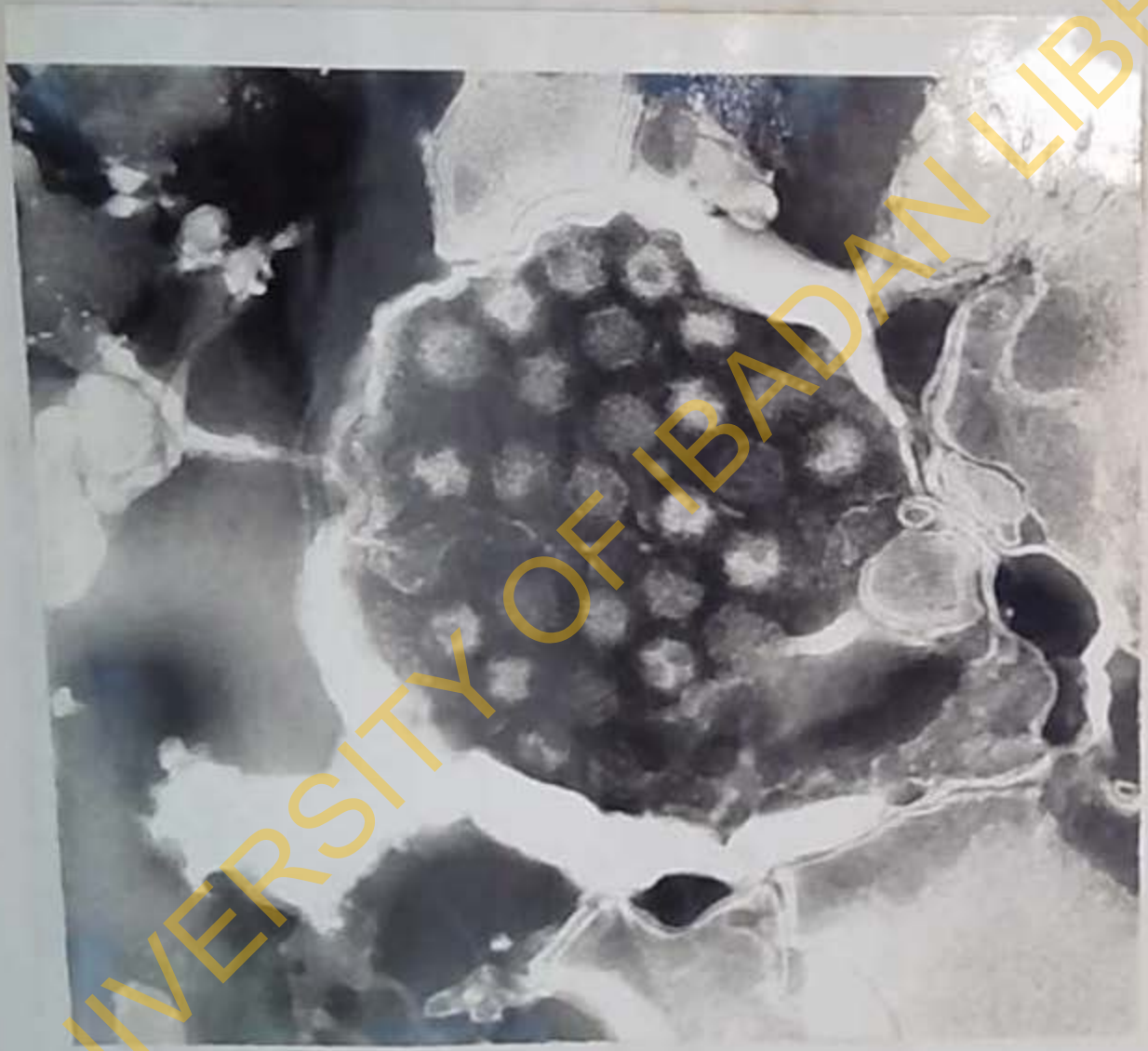
Thin section electron microscopy of infected mouse brain, revealed identical viral particles and associated structures as shen in infected BHK-21 cell cultures. Viral particles were intimately associated with granular viral matrix (Plate 6).

4.3 Reaction to physical agents

4.3.1 Thermal inactivation of Orungo virus

A 10% suspension of Orungo virus infected newborn mouse brain tissue (mouse brain antigen) was prepared in either phosphate buffered saline (PBS) pH 7.4 without serum or PBS supplemented with 5% of heat inactivated FCS. The virus suspension was centrifuged at 2500 rpm for 15 minutes and the supernatant fluid filtered through a "Millipore" membrane of APD 450 nm. The resulting filtrate was used for thermal inactivation studies. Two ml volumes were dispensed into rubber stoppered pyrex tubes (13 x 100 mm). These were immersed in a water bath at 56°C, 37°C, or placed on a laboratory bench or in a refrigerator (4°C) respectively. At designated intervals, tubes were removed and chilled in an ice bath and portions of the contents immediately titrated. The remaining portions were stored at -70°C for later testing for CF activity. In repeat experiments, virus aliquots were removed at

Plate 5



Orungo virus particles variously penetrated by the negative contrast medium (potassium phosphotungstate) X111,100.

Plate 6

Virus particles and granular matrix in the cytoplasm of a neuron in brain of moribund suckling mouse. The particles are embedded within the matrix. X64,000.

designated intervals following thorough mixing of a virus pool in a master tube placed at the respective temperatures and checked for infectivity and CF activity.

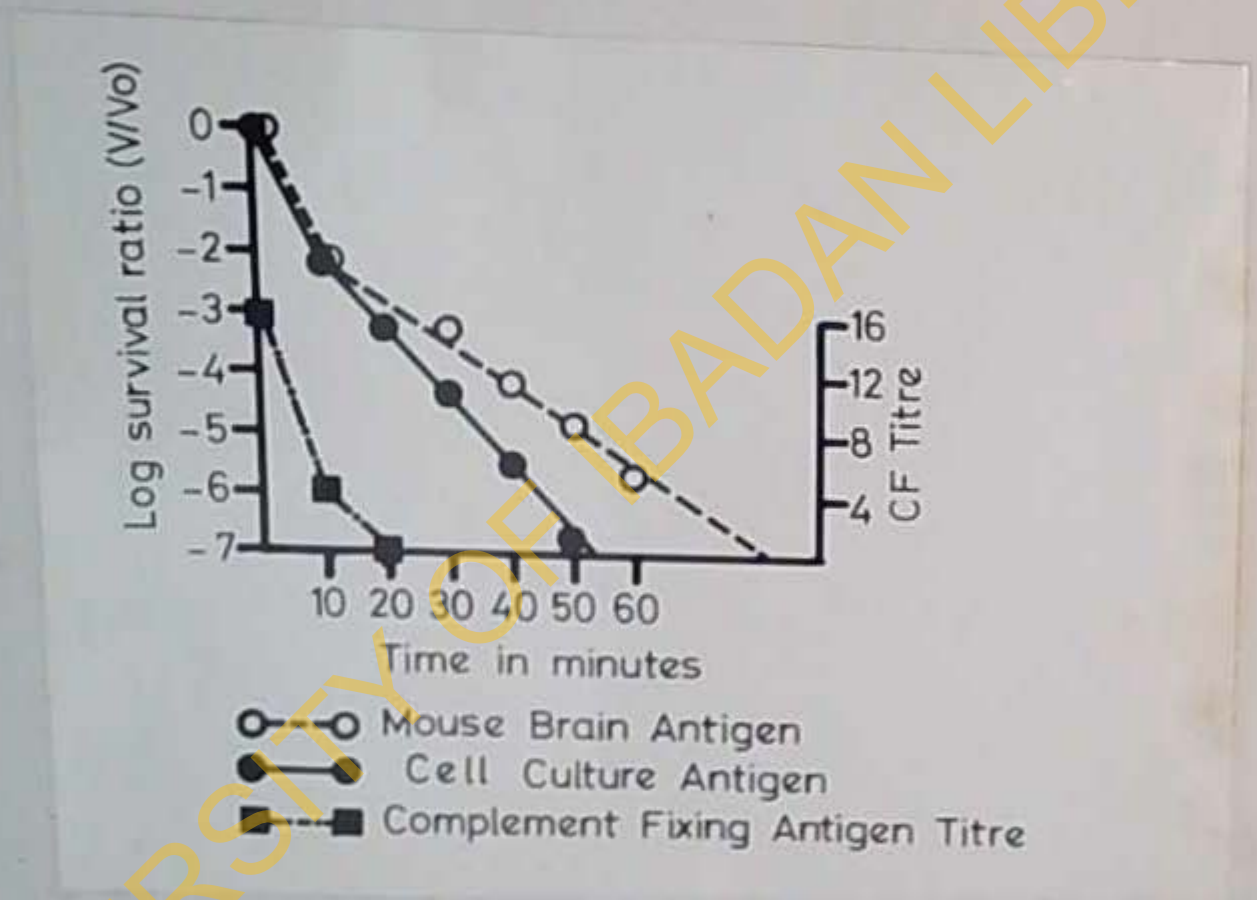
Virus grown in Vero cell culture (cell culture antigen) was also used in the thermal inactivation studies, however virus suspension was prepared in PBS only without serum addition and inactivation was carried out at 56°C only.

Thermal inactivation curves of Orungo virus at 56°C, 37°C and room temperature (22°C±2°C) are shown in Figs. 2, 3 and 4. There were two component curves of thermal inactivation of Orungo virus following inactivation at the three temperatures. Each component curve followed first order kinetics. The period of the more rapid inactivation phase was 10 minutes at 56°C, and 24 hours for both 37°C and room temperature. When the virus suspending medium was PBS only, the half-life of Orungo virus was 8 minutes at 56°C, 12 hours at 37°C and 15 hours at room temperature. On the addition of 5% serum, the corresponding periods for the half-life of Orungo virus was unchanged at 56°C, but 22 hours and 20 hours at 37°C and room temperature (22°C) respectively.

After 14 days at 4°C, 2 dex and 0.8 dex of virus infectivity were lost in virus suspended in PBS and PBS supplemented with 5% serum respectively.

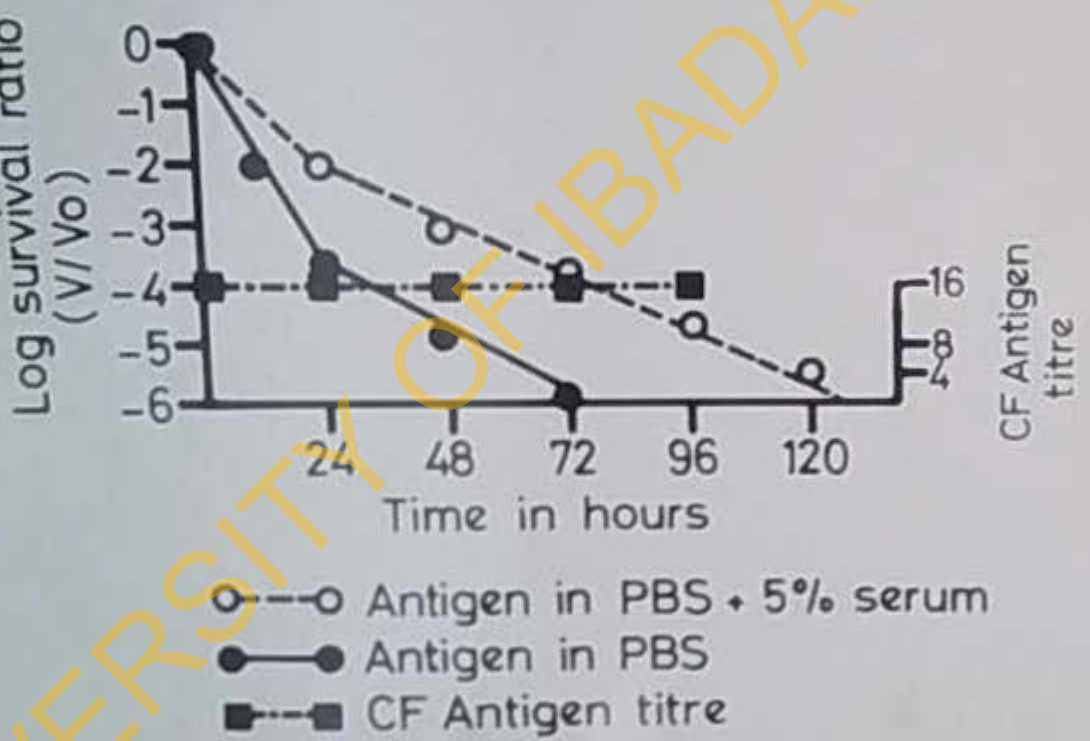
The CF activity of Orungo virus was reduced to undetectable levels in 60 minutes at 56°C. Inactivation at 37°C, 22°C and 4°C

Fig. 2



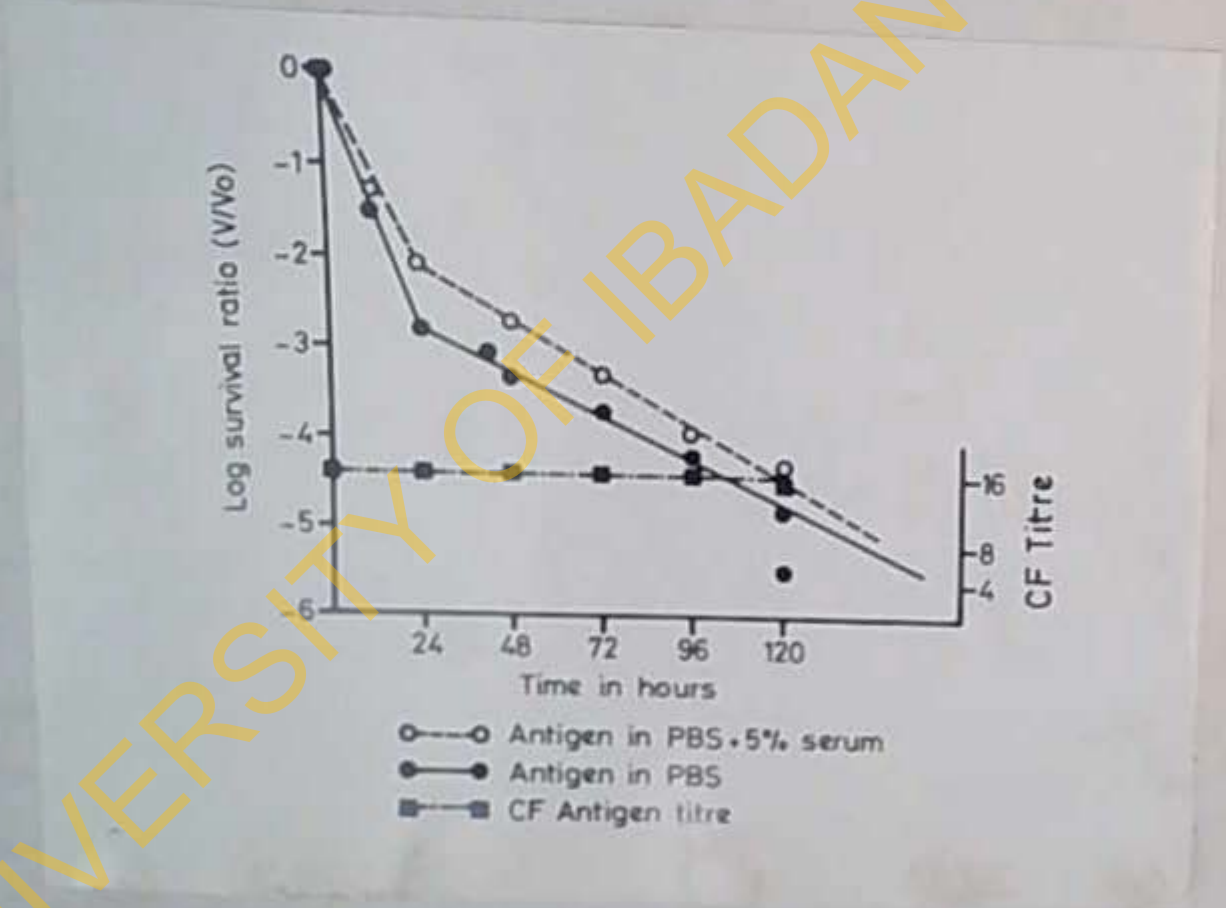
Thermal inactivation rate of Orungo virus at 56°C.

Fig. 3



Thermal inactivation rate of Orungo virus at 37°C.

Fig. 4



Thermal inactivation rate of Orungo virus at 23°C.

had no effect on the CF activity of Orungo virus.

4.3.2 Stability of lyophilized Orungo virus suspension

A 20% suspension of Orungo virus in PBS supplemented with 5% fetal calf serum was dispensed in 1.0 ml aliquots into 3 ml ampoules. The contents were shell-frozen by swirling in a dry ice-alcohol bath and left submerged until all ampoules had been prepared. Each ampoule was then transfered to the exhaustion manifold of an Edwards Freeze Dryer, Model EF 03 for evacuation. Evacuation was allowed to proceed until all material was dry. The ampoules were then sealed under vacuum and stored at 4°C. At intervals of 1, 3, 6, 12 and 18 months contents of three ampoules were each reconstituted with 1 ml of sterile distilled water and titrated by intracerebral inoculation of baby mice. Table 5 shows results of infectivity titrations of ampouled virus preparations. On lyophilization, i.e. day zero post lyophilisation 0.8 dex of virus was lost, a further loss of 1.0 dex was observed on storage at 4°C for 18 months. No virus was detected in unlyophilized samples stored for 3 months under the same condition.

4.3.3 Inactivation of Orungo virus by ultra-violet (UV) irradiation

A 10% suspension of Orungo virus in BAPS was clarified by centrifugation and filteration through a "Millipore" membrane. Twenty ml

TABLE 5

INFECTIVITY OF LYOPHILIZED ORUNGO VIRUS STORED AT 4°C.

Virus preparations	Titre (in dex) after indicated interval					
	0	1 mth.	3 mths.	6 mths.	12 mths.	18 mths.
Lyophilized	4.7*	4.6	4.2	4.0	3.8	3.7
Not lyopholised	5.5	2.9	0	-	-	-

of the filtered virus suspension was exposed in a petri dish (internal diameter 11 cm) to a Philip UV lamp (30W) placed at different distances from the suspension. The virus suspension was gently agitated by a magnetic stirrer. At given intervals 1 ml aliquots of virus suspension were removed and titrated immediately for infectivity and the rest stored at -70°C until tested for CF activity.

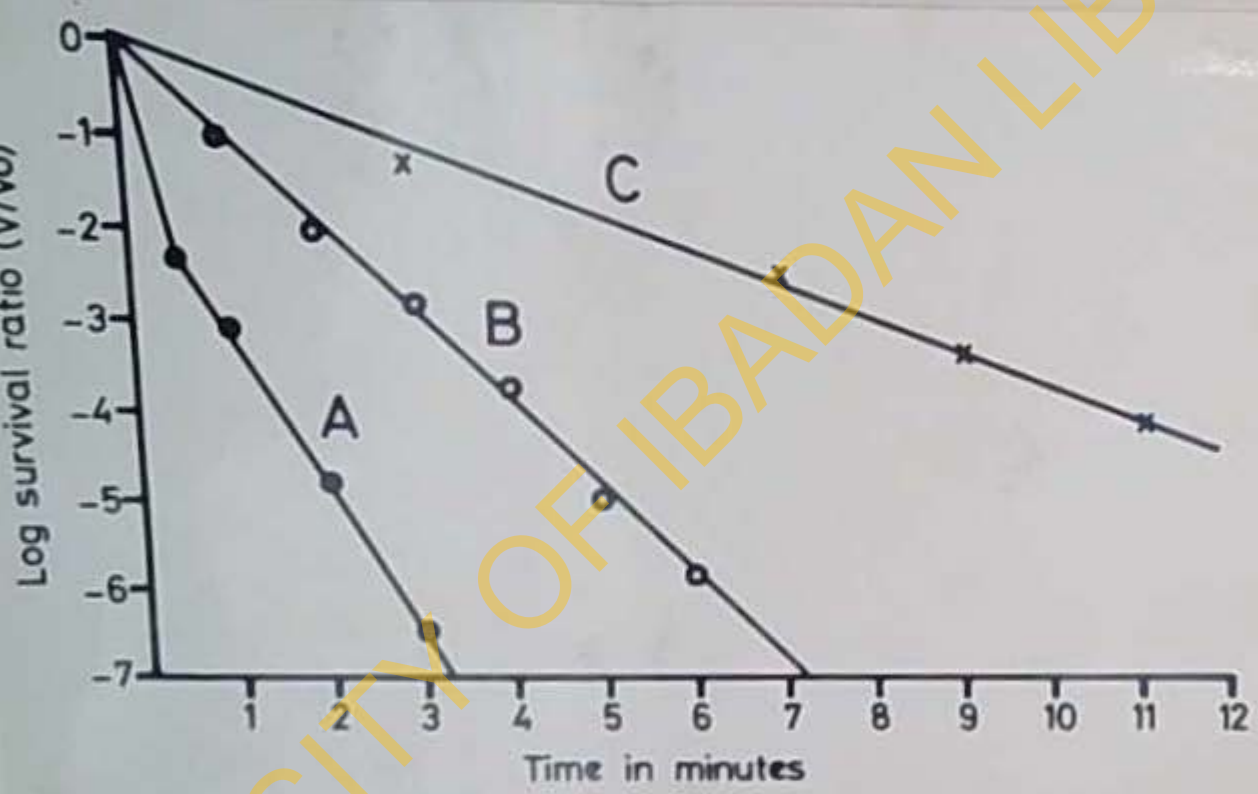
The effect of UV irradiation on the infectivity of Orungo virus are shown in Fig. 5. The half-life of Orungo virus with UV lamp source at distances of 10 cm, 25 cm, and 40 cm was 25 seconds, 105 seconds and 282 seconds respectively. CF antigen was not affected for as long as 20 minutes when the experiment was terminated.

4.4 Reactions to chemical agents.

4.4.1 Lipid solvent sensitivity

Ether (anesthesia grade) and chloroform (reagent grade) sensitivities of Orungo virus were determined by the method of Andrewes and Horstmann (1949) and a modification of the method of Feldman and Wang (1961) respectively. A 10^{-1} dilution of virus propagated in mouse brain was prepared in BAPS. For the other sensitivity test, virus preparation was held with ether (2:1) for

Fig. 5



Inactivation of Orungo virus by ultraviolet radiation; A = UV lamp 10 cm from virus suspension, B = UV lamp 25 cm from virus suspension, and C = UV lamp 40 cm from virus suspension.

16 to 20 hours at 4°C. For the chloroform test, virus preparation was thoroughly mixed in a final chloroform concentration of 1:10. Serial dilutions for inoculation were made in BAPS after removal of the solvent by evaporation (ether) or centrifugation (chloroform). Sodium deoxycholate testing was performed according to a modification of the method of Theiler (1957). Infected mouse brain suspensions were prepared in BAPS (10% V/V) and centrifuged at 10,000 rpm for 1 hour. The resulting supernatant fluid was mixed with equal volumes of 1:500 dilution of sodium deoxycholate. The control was prepared similarly but with BAPS diluent replacing the sodium deoxycholate. After incubating these mixtures at 37°C for 1 hour, they were diluted in serial ten-fold steps and inoculated. Known lipid solvent sensitive and resistant viruses were also included in the determinations.

On treating Orungo virus with ether, chloroform and sodium deoxycholate, there was 1.6 dex, 1.7 dex and 1.4 dex of virus infectivity reduction respectively (Table 6). Togaviruses of which yellow fever virus is a member are known to be unequivocally sensitive to the lipid solvents. This is borne out by the results obtained following the treatment of yellow fever, with lipid solvents. On the other hand, Lebombo virus of the orbivirus taxon, (Borden et al. op. cit.) shows a typical reaction of the taxon which are known to be relatively stable to lipid solvents.

TABLE 6

LIPID SOLVENT RESISTANCE OF ORUNGO, YELLOW FEVER AND LEBOMBO VIRUSES.

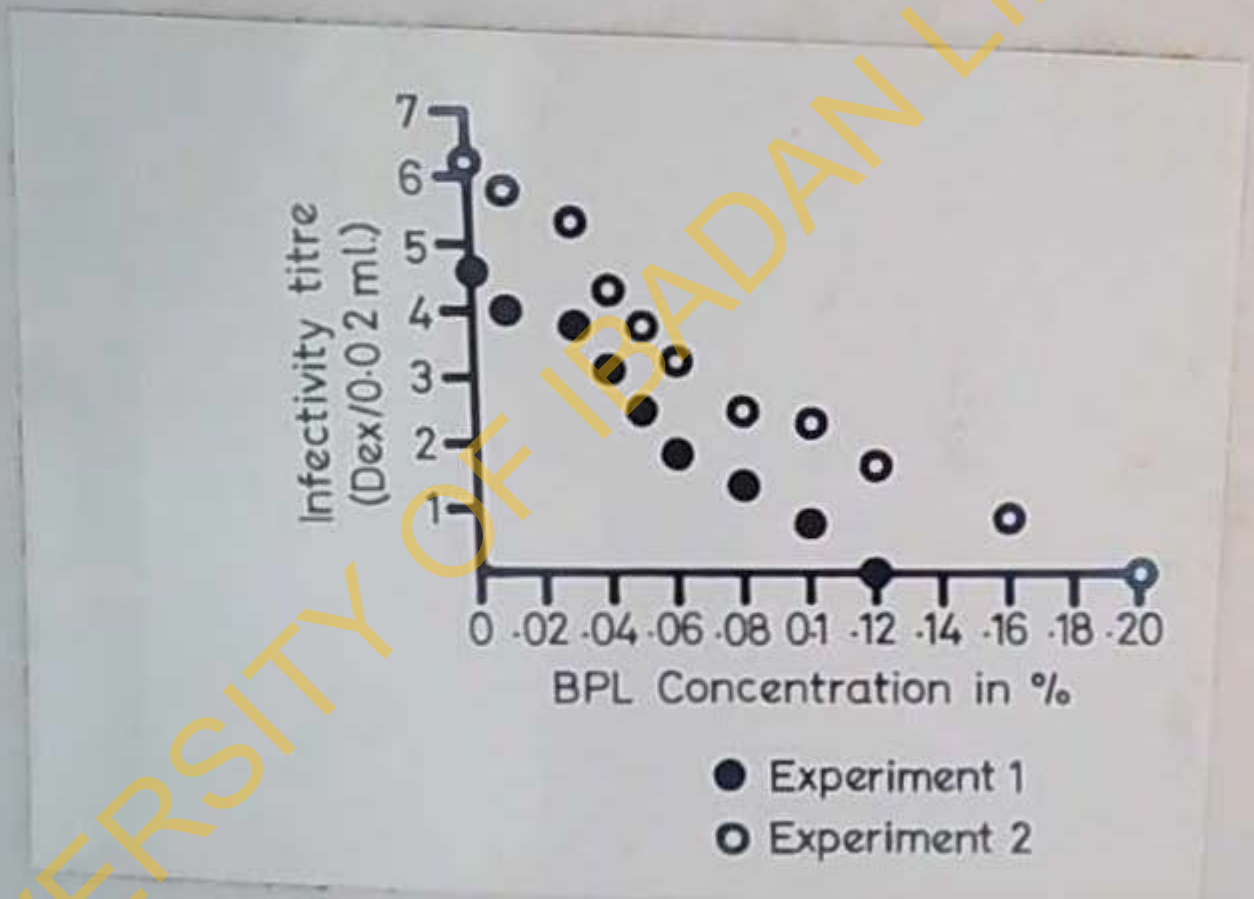
	INFECTIVITY REDUCTION (IN DEX/0.02 ml.)		
	ETHER	CHLOROFORM	SODIUM DEOXYCHOLATE
ORUNGO	1.6	1.7	1.4
YELLOW FEVER	3.8	4.1	5.3
LEBOMBO	0	0.2	0.4

4.4.2 Beta-propiolactone (BPL) inactivation of Orungo virus

Infected mouse brain material was triturated with cold normal saline to make a 20% suspension. The suspension was clarified by centrifugation and the pH of supernatant fluid was adjusted to 8, and mixed with an equal volume of prepared dilutions of BPL in order to obtain BPL concentration range of 0.01% to 0.3%. Virus suspensions in this experiment were thus reduced to 10%. In one series of experiments, the BPL-virus suspension was stored at 4°C for 4 days with occasional agitation, before titrating in baby mice for infectivity. For the second series, inactivation was carried out at 37°C, and samples for infectivity titration removed at 5 minute intervals. Aliquots were also stored for CF test.

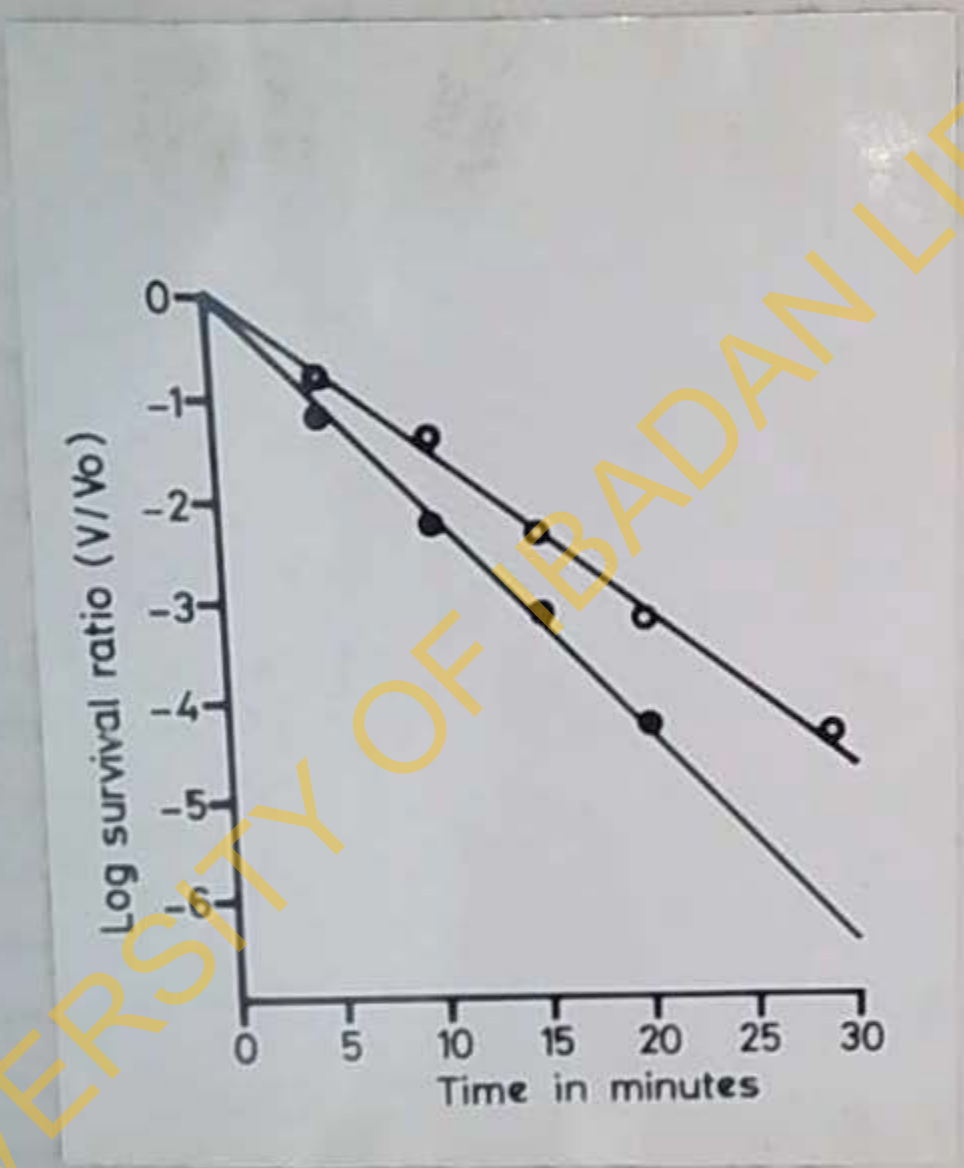
The results of BPL inactivation of Orungo virus are shown in Figs. 6 and 7. BPL reduced Orungo virus titre from 6.3 dex to non-detectable levels in 4 days at 4°C with a 0.2% drug concentration. On the other hand, a 0.12% drug concentration of BPL was required to completely inactivate 4.6 dex of the virus under the same condition. At 37°C, no significant difference was found in the inactivation rate of Orungo virus by either 0.3% or 0.2% drug concentration. The half-life of Orungo virus at either concentration was 7.5 minutes. At 0.1% drug concentration, half-life of Orungo virus was 11 minutes.

Fig. 6



Beta-propiolactone (BPL) inactivation of Orungo virus at 4°C for 4 days.

Fig. 7



Beta-propiolactone (BPL) inactivation of Orungo virus at 37°C. (Open circles = 0.1% BPL; closed circles = 0.3% BPL).

4.4.3 Formalin-inactivation of Orungo virus

Inactivation studies with formalin were carried out under similar conditions as for BPL using final concentrations of 0.01% and 0.01% formalin. Inactivation was carried out at 4°C for 4 days only. The source of formalin was commercial formaldehyde at a 40% formalin concentration.

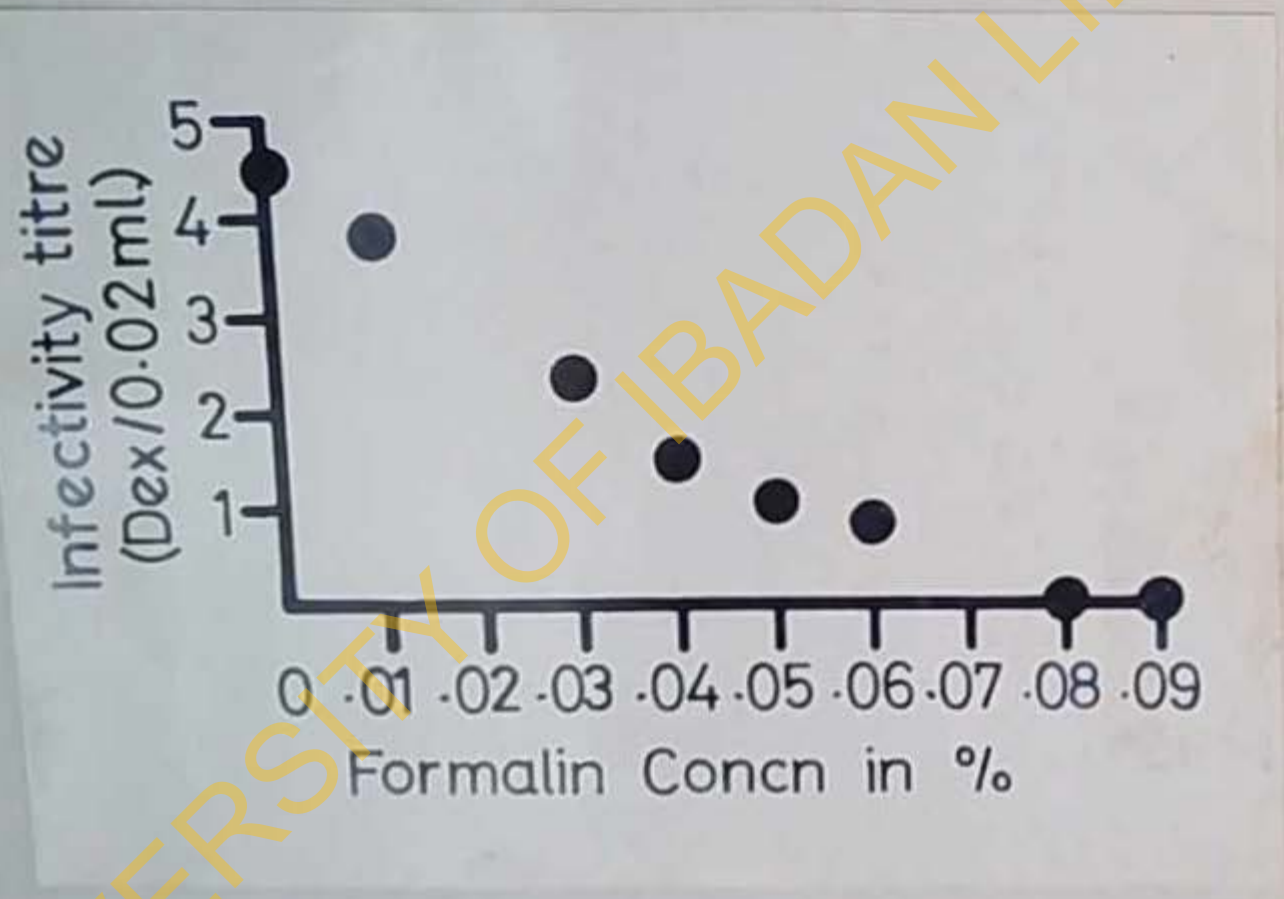
The inactivation of Orungo virus is shown in Fig. 8. Under the conditions of the experiment, 4.5 dex of the virus was reduced to undetectable level by 0.08% formalin concentration.

4.4.4 pH stability of Orungo virus

Inactivation of BHK-21 cell culture adapted virus was determined at various hydrogen-ion concentrations ranging from pH 3.0 to pH 10.0. Diluents of differing hydrogen-ion concentrations were prepared by adjusting 0.4% bovine-plasma albumin in distilled water with 0.1N NaOH and 0.1N HCl. Samples of infected BHK-21 cell-cultures were diluted 1:10 in each pH solution and incubated for 3 hours at room temperature (22 ± 1°C). Ten-fold dilutions of each sample were prepared in Hank's balanced salt solution (HBSS) containing 5% inactivated fetal calf serum and titrated by IC inoculation of baby mice. Controls consisted of infected cell culture stored at -70°C until tested.

Orungo virus infectivity titers were stable between pH 5.0

Fig. 8



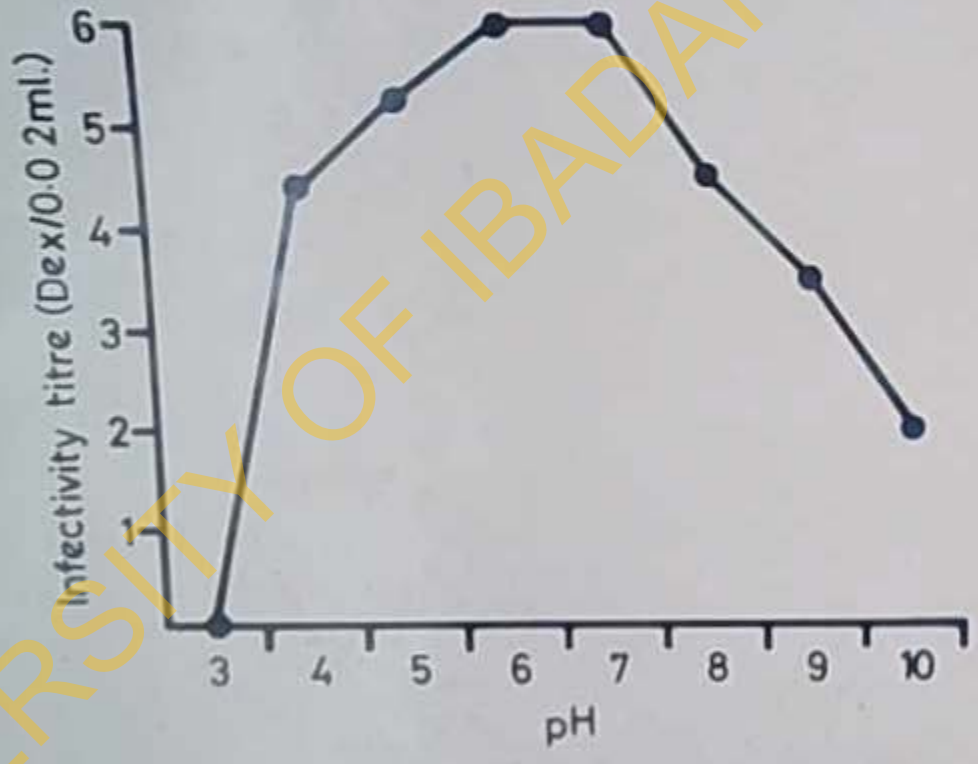
Formalin inactivation of Orungo virus at 4°C for 4 days.

and pH 7.0, less than 1 dex reduction in virus titre occured at these pH range (Fig. 9). Virus was undetectable at pH 3.0, but titered 4.3 dex after treatment at pH 4.0. In the alkaline pH range, virus titers were 4.6 dex, 3.5 dex and 2.2 dex for pH 8.0, 9.0 and 10.0 respectively. Control titre was 6.0 dex.

4.4.5 Effect of 5-iododeoxyuridine (IUDR) on Orungo virus multiplication

The effect of 5-iododeoxyuridine on Orungo virus multiplication was studied in BHK-21 tube culture by procedures previously outlined by Liebhaber et al. (1965). Herpes virus (DNA) and eastern encephalitis (EE) virus (RNA) were employed as control viruses of known nucleic acid composition. A 10^{-2}M stock of IUDR was prepared in distilled water; to achieve solubility 0.1N NaOH was added to raise the pH to 9.0 and the solution was heated briefly to 75°. Cultures were incubated for 4-5 hr. with maintenance medium containing 10^{-4} IUDR solution, drained free of this medium and inoculated with virus and then refed with fresh maintenance medium containing IUDR. Fluids were changed daily. Non-IUDR-treated cultures served as controls. All cultures were harvested when the controls showed at least a 3+ cytopathic effect (CPE), which was 48 hours post-inoculation. The cells and fluids were frozen and thawed twice and assayed for virus in BHK-21 tube cultures using 4 tubes/dilution.

Fig. 9



Infectivity titre of Orungo virus at different pH values.

In the presence of 10^{-4}M IUDR concentration the yeild of EE (control RNA virus) and Orungo virus was not reduced (Table 7). A reduction of 6.1 dex oonured when Herpes (control DNA virus) was similarly treated. Orungo virus may therefore be an RNA virus.

4.5 Biological characteristics of Orungo virus in experimental hosts

4.5.1 Vertebrates

4.5.1.1 Swiss albino white mouse

Since primary isolations of Orungo virus were carried out in Swiss albino white mice, it was essential to determine certain basic biological characteristics of Orungo virus for this host. Investigations were conducted on the effects of dose on virus, route of inoculation and age of host on the course of Orungo virus infection.

Prior to commencement of these studies, the infectivity titer of each of the Orungo virus strains was determined in 2-3 day old baby mice. Serial ten-fold dilutions of each virus pool were made, and 0.02 ml of each dilution inoculated IC into each of six 2-3 day old suckling mice in a litter. The inoculated mice were observed for signs of illness for 14 days. End points were calculated by the method of Reed and Muench (op. cit.) and expressed in dex (= log 10, Haldane, op. cit.).

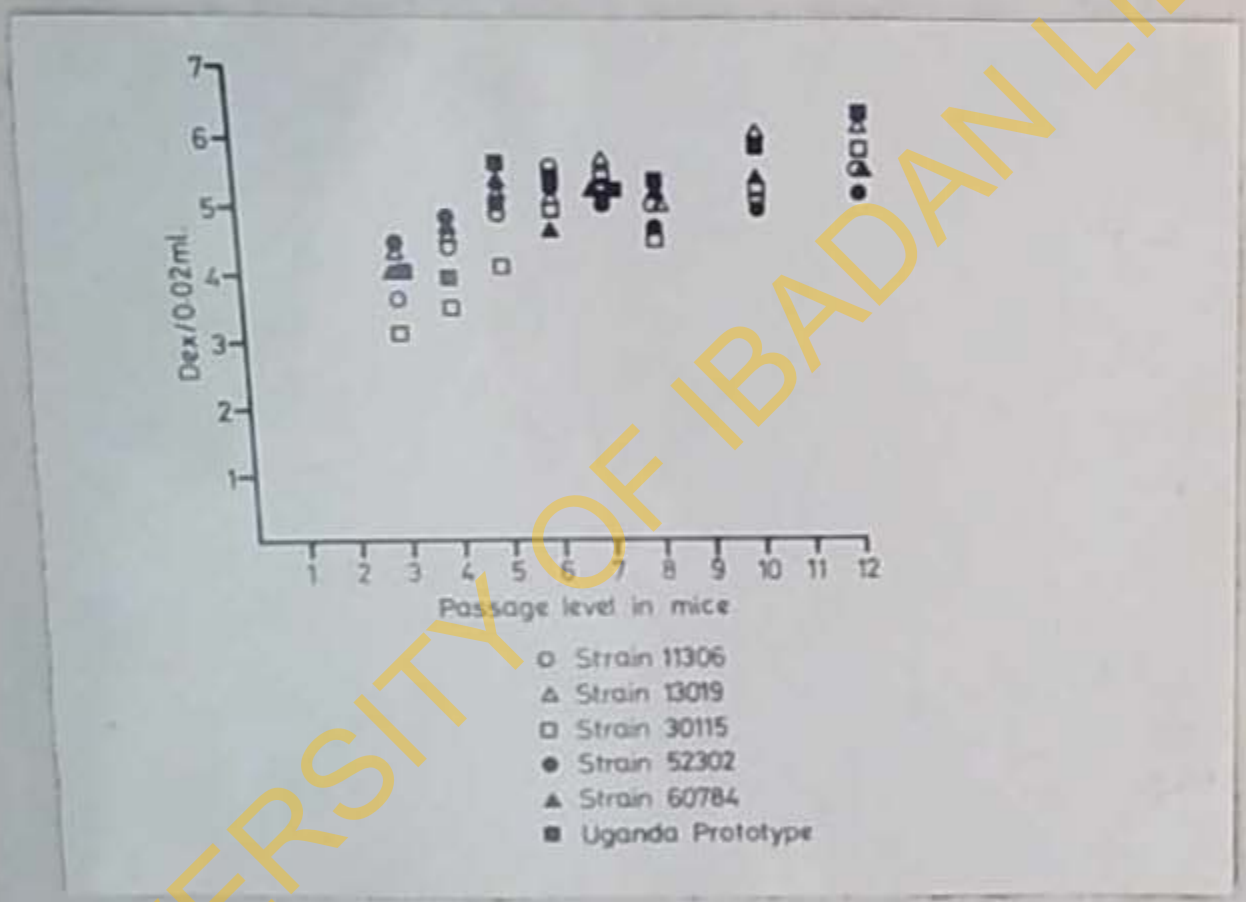
The results of infectivity titer are shown in Figure 10. Only 2 strains, 13019 and 52302 had infectivity titers above 4 dex at

TABLE 7

EFFECT OF 5-IODODEOXYURIDINE (IUDR) ON MULTIPLICATION OF ORUNGO VIRUS IN BHK-21 CULTURES

Virus	Inoculum	Virus Yield (dex $TCID_{50}$/ml.)	
	(total dex $TCID_{50}$)	No. IUDR	10^{-4} IUDR
Orungo	3.0	5.9	6.1
EE	4.5	9.9	10.2
Herpes	5.0	8.2	2.1

Fig. 10



Infectivity of Orungo virus strains following successive mouse intracerebral passages.

mouse brain passage 3. However further mouse intracerebral passages showed increasing infectivity titers until the sixth passage when all the six strains had titers above 4 dex, and became stabilized for the experimental animal. Although there were general increases in titers between the 10th and 12th IC passage, however when considered for each virus strains, these increases are not significant.

Further determination of infecticity was measured by the length of time inoculated mice survive known doses of virus inoculum. This is known as the average survival time (AST). The AST is calculated as - Sum of mice surviving per day for the duration of the test divided by the original number of mice inoculated.

For example, if 12 mice were inoculated, and all of them survived for the first three days, 6 died on day 4, and no survivors on day 5,

$$\text{The AST} = \frac{\text{the sum of mice surviving per day till close of experiment}}{\text{Number of mice originally inoculated}}$$

$$= \frac{12 + 12 + 12 + 6 + 0}{12}$$

$$= \frac{42}{12}$$

$$= 3.5 \text{ days.}$$

Any death occuring within the first 24 hours after virus inoculation was not taken into account in calculating the AST.

The AST of 2-3-day old mice inoculated with any of the strain of Orungo virus was 3.5 and 3.1 days for $10LD_{50}$ and $100LD_{50}$ virus dose respectively. The amount of virus in brain harvests as measured by infectivity titration decreases with increasing age at inoculation time of the mice. This was accompanied by a corresponding increase in the AST of inoculated mice. No virus was detected in the brains of 14-day old mice sacrificed at the close of experiment (Table 8).

4.5.1.2 Effect of Orungo virus infection on the growth of Swiss albino mice

It was observed from the preceeding experiment that 10-day old mice inoculated IC with 10 LD_{50} of Orungo virus exhibited two types of reactions: (a) became sick and succumbed to infection; or (b) became sick, recovered but remained unthrifty through the course of the experiment. The course of Orungo virus infection was therefore studied in greater detail in 2- and 10-day old mice inoculated IC with 10 LD_{50} of Orungo virus. Controls, uninoculated animals and those inoculated IC with sterile diluent were set in parallel. Following inoculation, all baby mice were pooled and sorted out into approximate equal weights and distributed in groups of six per mother. Inoculated animals were observed to onset and course of illness and feeding habits. In addition the effect of

TABLE 8

AGE RELATED INFECTIVITY TITRES IN BRAIN HARVESTS AND AST OF SWISS ALBINO MICE INOCULATED WITH ORUNGO VIRUS BY THE IC ROUTE

AGE OF MICE IN DAYS	INFECTIVITY TITERS AND AST FOLLOWING INFECTION WITH			
	10 LD_{50} VIRUS		100 LD_{50} VIRUS	
	DEX/0.02 ml.	AST	DEX/0.02 ml.	AST
1	8.5	3.3	8.5	3.1
2	8.6	3.5	8.6	3.1
3	8.5	3.8	8.6	3.4
4	8.3	4.5	8.3	3.6
5	8.1	4.6	8.2	4.0
6	7.6	5.5	7.6	4.4
7	6.9	6.1	6.3	4.9
8	6.2	7.4	6.3	6.6
9	6.2	8.0	6.8	6.5
10	5.2	> 14.0	5.3	6.6
11	5.5	> 14.0	4.5	9.1
12	4.9	> 14.0	4.5	9.1
13	3.6	> 14.0	4.2	11.9
14	0	> 14.0	0	>14.0

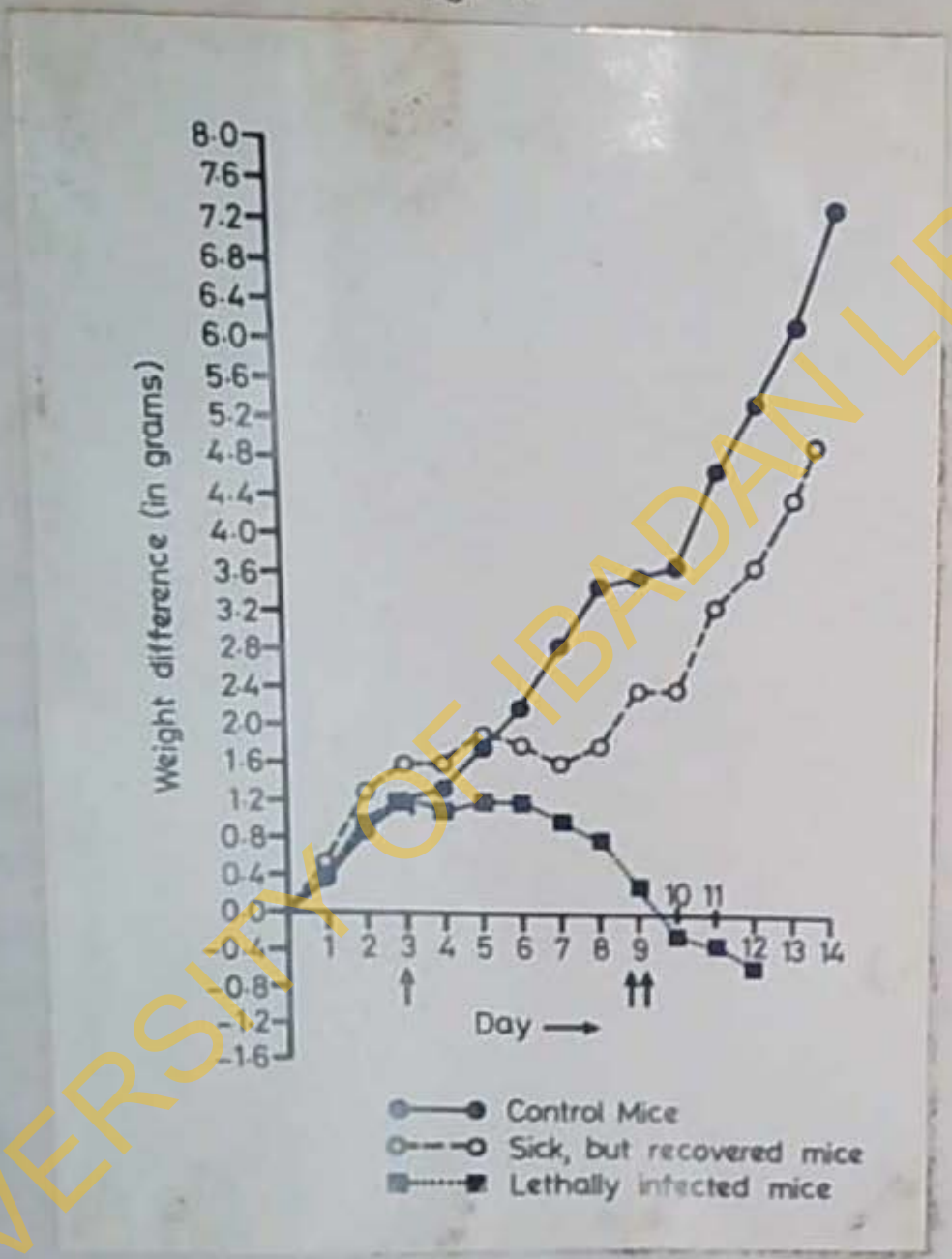
Orungo virus infection on the growth of 10 day old mice was studied by daily recordings of weight gains.

Onset of illness in 2-day old mice was from day 2 and death occurred within 24 - 36 hours. Sick mice were hunched up, showed rapid respiration, but continued feeding until moribund. With 10 day old mice, onset of illness varied from 3-7 day post inoculation, and course of illness was up to 6-7 days ending terminally or in recovery. Sick animals showed ruffled hair, hypersensitivity to sudden noise and hind leg paralysis in animals that succumbed to infection. Although animals continued feeding until 2-3 days before death, there was a progressive wasting away with resultant loss in weight (Fig. 11). There was no significant difference in the weight gains of control uninoculated and animals inoculated with sterile diluent. Animals that became sick, but recovered never attained the same weight as control animals. There was an initial loss in weight for 2-3 days, followed by a slow daily increase in weight. Animals that succumbed to virus infection show a progressive weight loss until death. In general, the loss in weight was noticed a day earlier than the onset of clinical s symptoms.

4.5.1.3 Age susceptibility of Swiss albino white mice to inoculation with Orungo virus

Groups of mice from 1 day to 14-day old were inoculated by three different routes, intracerebral (IC), intraperitoneal (IP)

Fig. 11



Effect of intracerebral inoculation of Orungo virus on the development of 10-day old Swiss albino mice. Onset of illness (↑), death or recovery (↑↑).

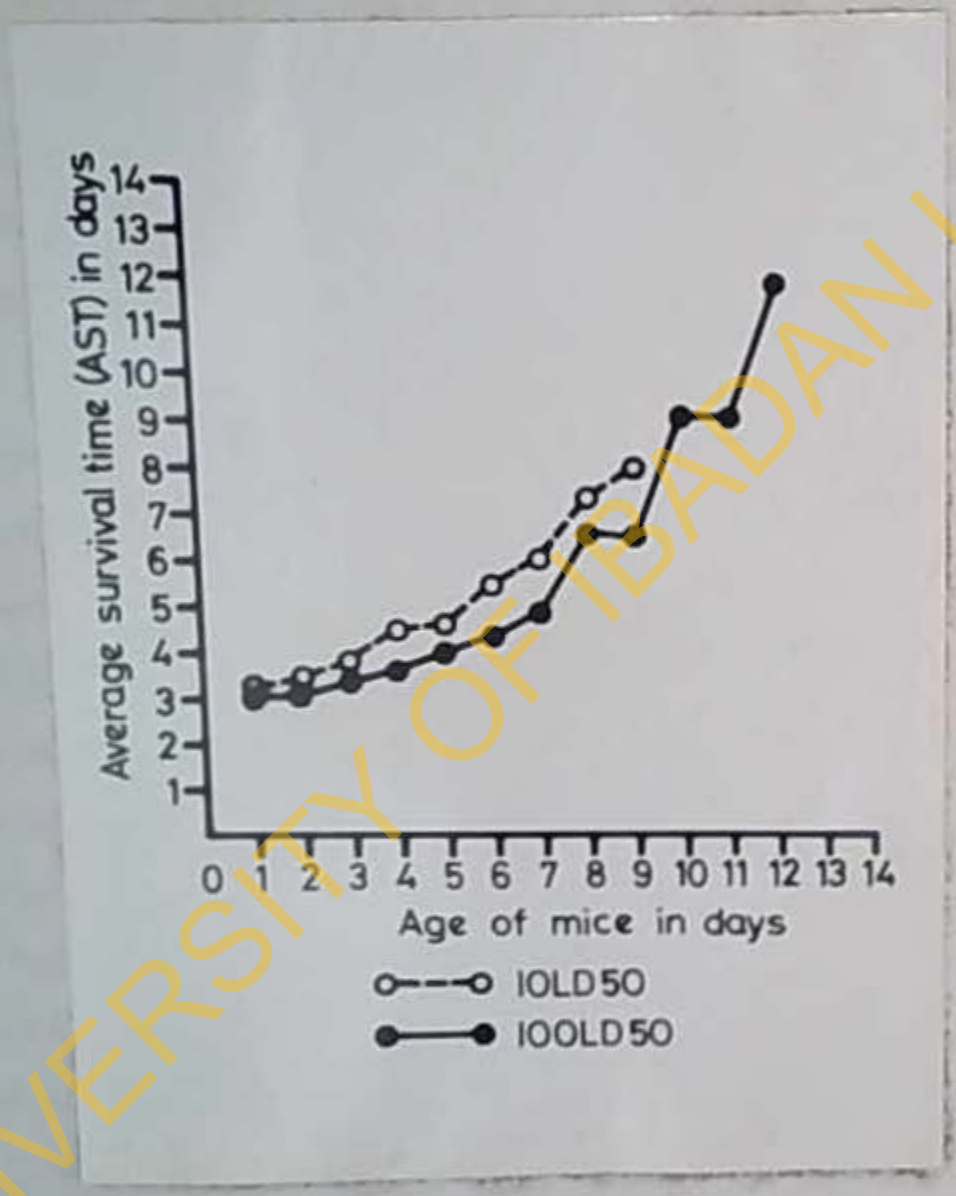
and subcutaneous (SC), with either 10 LD_{50} or 100 LD_{50} of Orungo virus. The mice were observed for 14 days, after which the AST was calculated. Sick or dying mice were harvested and two brain harvests from each group titrated individually. For this experiment, strain H13019 of Orungo virus was used.

All the mice survived inoculation by the IP or SC route with 100 LD_{50} or 10 LD_{50} of Orungo virus, and sensitivity to lethal infection by the IC route decreased with age. The AST of newborn mice inoculated IC with 100 LD_{50} and 10 LD_{50} of Orungo virus was 3.1 days and 3.3 days respectively (Fig. 12). Ten day old mice succumbed to IC infection of 100 LD_{50} of virus with an AST of 9.1 days, however only 18 or 48 mice of the same age group infected IC with 10 LD_{50} of virus survived. The thirty mice that died had an AST of 12.5 days. Mice 14 days old and older were not killed by either 10 LD_{50} or 100 LD_{50} of Orungo virus inoculated IC.

4.5.1.4 Organ distribution of Orungo virus in mice

Six litters of 2-3-day old baby mice were inoculated IC with either 10 LD_{50} or 100 LD_{50} of Orungo virus. Daily, two inoculated mice were sacrificed and the following organs: brain, heart, lung, liver, kidney and spleen removed for virus titrations. In addition blood and urine were also assayed for virus content. Each organ was washed in BAPS to remove as much blood as possible. From a 10%

Fig. 12



Age susceptibility of Swiss albino mice to Orungo virus intracerebral infection.

suspension of each organ (W/V) or fluid (V/V), in BAPS, serial ten-fold dilutions were made and titrated by IC inoculation of 2-3-day old mice. Inoculated mice were observed for 14 days and virus infectivity titers calculated by the method of Reed and Muench (op. cit.). Brains of sick or moribund mice were removed and tested by CF test against Orungo MAF.

Ten-day old mice were similarly inoculated with 100 LD_{50} of Orungo virus. Distribution of virus in each organ was assayed as described for 2-3 day old mice. Strain Ib H13019 was used as the inoculum in all experiments.

Daily virus infectivity titers in the different organs are shown in Table 9. There was a progressive increase in amount of virus in the brain from day 1 post infection (p.i.) to day 6 p.i., which yielded the highest infectivity of 6.3 dex. No virus was detected in the heart, lung, liver and kidney on the first two days following infection. The peak infectivity for each of these organs was recorded on day 5 p.i. By day 6 p.i., the level of virus had fallen in each of the organs; no virus was detected in the kidney on this day. Low level viremia and viruria, occured on day 2 and day 3 p.i. The viremia reached a peak (2.8 dex) on day 4 p.i. and dropped to 0.5 dex on day 6 p.i. Mice became sick on day 4 p.i. which coincided with the day of peak viremia. No significant difference was found in the infectivity level of urine

TABLE 9

INFECTIVITY TITRES (DEX/0.02 ml.) IN DIFFERENT ORGANS OF 2-3-DAY OLD BABY MICE INOCULATED WITH 100 LD_{50} OF ORUNGO VIRUS (STRAIN IB H13019). BY THE INTRACEREBRAL ROUTE

Day post infection	Brain	Heart	Lung	Liver	Kidney	Spleen	Urine	Blood
1	1.1	0	0	0	0	0	0	0
2	2.0	0	0	0	0	0	0	0
3	4.3	2.2	3.2	2.1	2.1	0	0.5	0.9
4	4.8	2.7	2.9	1.6	2.0	0	0.7	2.8
5	5.8	3.4	3.5	2.3	1.1	1.1	0.7	1.7
6	6.3	2.5	1.9	2.2	0	1.7	0	0.5

samples collected from day 3 p.i. to day 5 p.i., and no virus was detected in the urine sample collected on day 6 p.i. (Fig. 13).

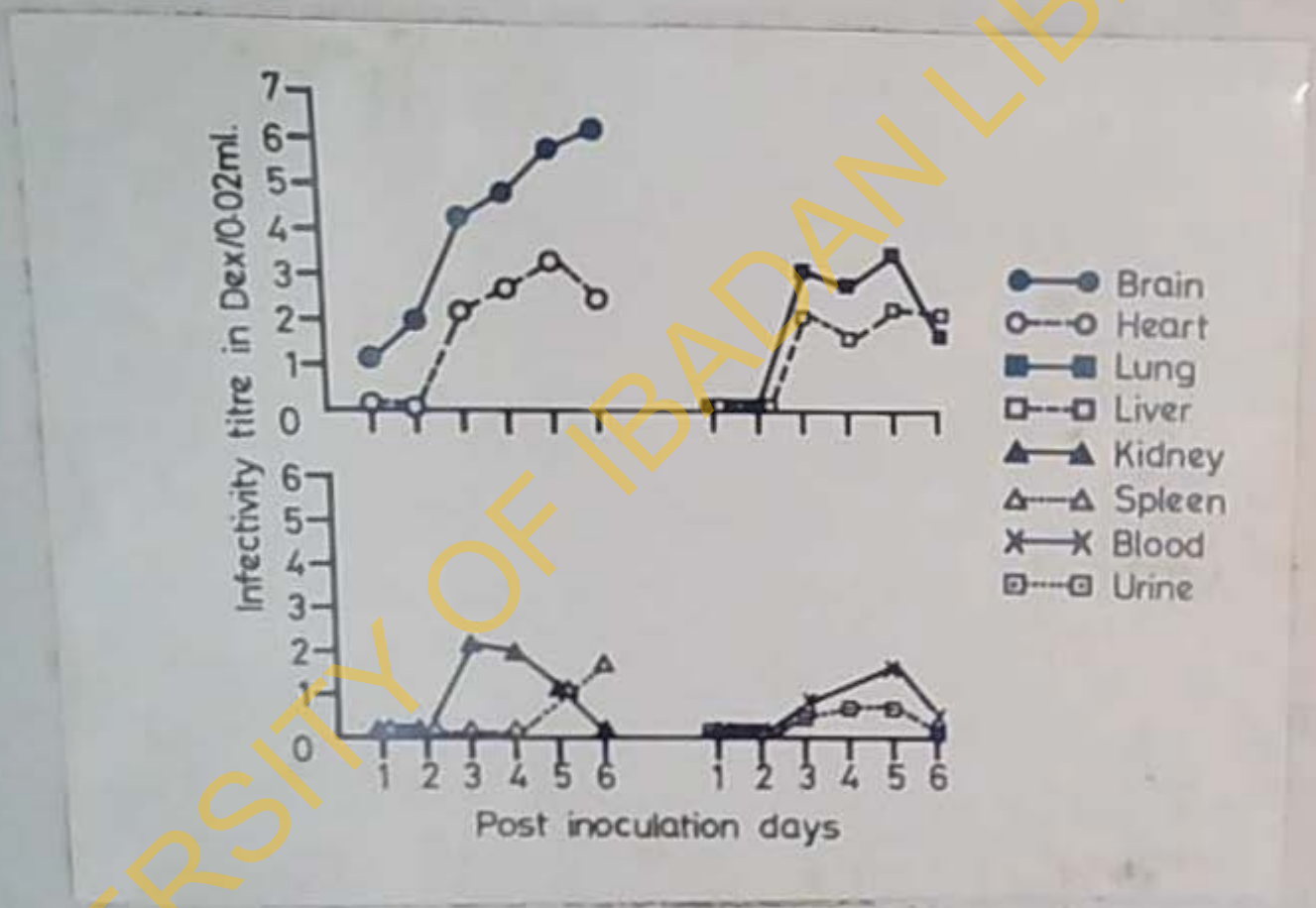
The viremia and viruria patterns were further studied in detail using two doses: (10 LD_{50} and 100 LD_{50}) of Orungo virus. In addition baby mice which became sick following inoculation with daily blood and urine sample collections were tested by CF with Orungo MAF. Table 10 shows the viremia and viruria patterns following infection with the two virus doses.

The level and duration of viremia as well as viruria were higher and longer when a lower level of virus was used. Similarly AST of mice inoculated with 10 LD_{50} of virus was 5.3 days as compared with 3.6 days following inoculation with 100 LD_{50} of virus.

Ten day old mice

Virus was detected in the brains of 10-day old mice infected with 100 LD_{50} of virus for the first eleven days post infection. The highest infectivity (3.2 dex) was obtained on day 6 p.i., thereafter there was a progressive reduction in infectivity until day 12 p.i. when no virus was detected (Table 11). Other organs: lung (day 2, 3 p.i.), heart (day 5 p.i.), blood (day 2 p.i.), and urine (day 11 p.i.) yielded low level virus infectivity titers (range 0.5 dex - 0.9 dex). No virus was detected in the liver, kidney and spleen throughout the duration of experiment. All

Fig. 13



Organ distribution of Orungo virus in 2-day old Swiss albino mice following intracerebral inoculation.

TABLE 10

VIREMIA AND VIRURIA PATTERNS IN 2-DAY OLD SWISS ALBINO MICE INFECTED WITH ORUNGO VIRUS

Days post inoculation	INFECTIVITY TITRE			
	INOCULUM = 10 LD_{50}		INOCULUM = 100 LD_{50}	
	Viremia titre	Viruria titre	Viremia titre	Viruria titre
1	0	0	1.5[a]	0
2	1.7	0	2.5	1.0
3	1.8	1.1	2.5	1.0
4	2.1	1.2	0.5	1.0
5	3.6	1.7	-[b]	-
6	0.6	0.7	-	-

a = infectivity titre in dex/0.02 ml.

b = no mice available, all died on the previous day.

TABLE 11

INFECTIVITY TITRES IN (DEX/0.02 ml.) IN DIFFERENT ORGANS OF 10-DAY OLD MICE INOCULATED WITH 100 LD_{50} OF ORUNGO VIRUS (STRAIN H13019)

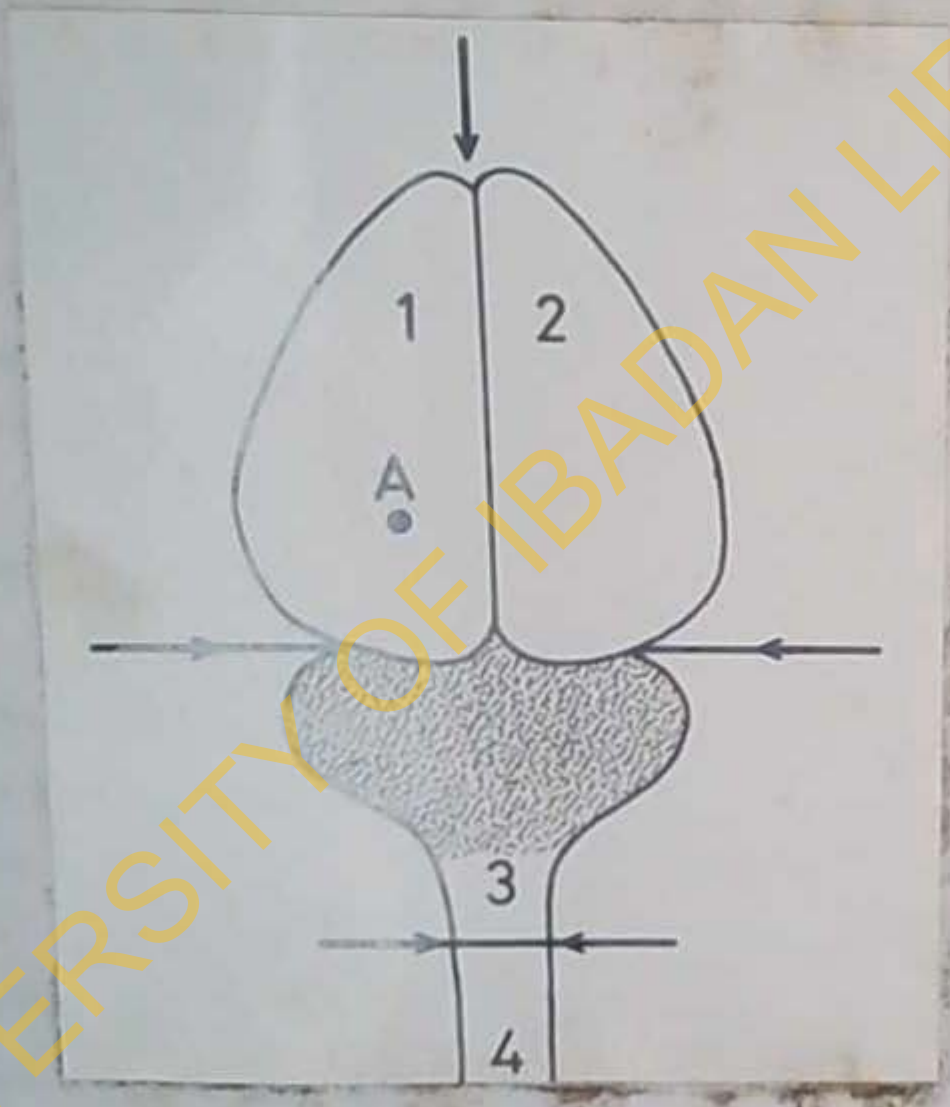
Days Post Infection	Brain	Lung	Heart	Blood	Urine
1	1.0	0	0	0	0
2	1.8	0.9	0	0.7	0
3	1.7	0.8	0	0	0
4	2.5	0	0	0	0
5	2.1	0	0.8	0	0
6	3.2	0	0	0	0
7	2.8	0	0	0	0
8	1.6	0	0	0	0
9	0.9	0	0	0	0
10	0.7	0	0	0	0
11	0.7	0	0	0	0.5
12	0	0	0	0	0
13	0	0	0	0	0
14	0	0	0	0	0

samples positive for virus infectivity test were also confirmed in CF tests. Ten day old mice that survived infection with Orungo virus were bled 28 days p.i. Serum samples from 2 mice each were pooled and tested for specific neutralising antibody to Orungo virus. Only mice inoculated by the IC route developed specific neutralizing antibody to Orungo virus. The range of neutralizing index was 1.2 dex - 1.6 dex.

4.5.1.5 Spread of Orungo virus in mouse brain

The spread of Orungo virus within the brain of 2-3 day old mice following IC inoculation into the routine site (Fig. 14) was investigated in baby mice inoculated with either 10 LD_{50} or 100 LD_{50} of Orungo virus. Two mice were removed daily, and each mouse brain aseptically removed and divided with a sharp razor blade along the longitudinal and transverse fissures, and below the cerebellum (Fig. 14). These divisions yielded the following brain sections: the right and left cerebral halves and the cerebellum with the medulla oblongata. The spinal cord was removed from the decapitated mouse, by first removing the hind legs and the tail and forcing the spinal cord with a jet of sterile diluent applied through a needle and syringe. Pooled sections of the brain were titrated in suckling mice.

Fig. 14



Mouse brain to show site of inoculation (A) and lines of divisions into left cerebrum (1), right cerebrum (2), cerebellum with medulla oblongata (3) and spinal cord (4).

The distribution of Orungo virus in the different se'tions of the mouse brain is shown in Tables 12 a & b. No significant differences were detected in the amount of virus found in the left and right cerebral halves. The level of infective virus in the cerebellum was consistently lower than in either of the cerebral halves. The spread of virus to the spinal cord was detected only when the higher infecting virus dose was used. The complem nt fixing antigen developed in parallel with the virus infectivity.

4.5.1.6 Histopathologic and immunofluorescent studies of mice infected with Orungo virus

Two, ten and twenty-one-day old mice were inoculated IC with Orungo virus (strain Ib H13019). Mice were killed serially at various intervals after inoculation and processed in the following manner: The mice were trimmed of appendages and skin, except on the midline, they were then split longitudinally through the midline with a sharp razor blade. One half was transferred to Rossman's fluid (9 parts of alcoholic saturated solution of picric acid to 1 part of formaldehyde) for fixation and subsequent routine processing in an automatic tissue processor for histopathologic examination. The other half was mounted in a cryostat tissue embedding medium and left to freeze at -18°C to -20°C prior to sectioning for immunofluorescent examination.

TABLE 12a

DEVELOPMENT OF VIRUS INFECTIVITY AND CF ANTIGEN IN DIFFERENT SECTIONS OF THE BRAIN FOLLOWING INOCULATION OF 2-3-DAY OLD SWISS ALBINO MICE WITH 10 LD_{50} OF ORUNGO VIRUS.

Days Post Infection	WHOLE BRAIN		LEFT CEREBRUM		RIGHT CEREBRUM		CEREBELUM		SPINAL CORD	
	Infectivity	CF	Infectivity	CF	Infectivity	CF	Infectivity	CF	Infectivity	CF
1	0	0	0	0	0	0	0	0	0	0
2	0.5*	0	0	0	0	0	0	0	0	0
3	0.9	2**	0.6	0	0.9	0	0	0	0	0
4	1.6	8	1.4	2	1.3	4	0	0	0	0
5	3.0	16	2.7	4	2.1	4	0.7	+***	0	0
6	4.5	16	3.5	8	2.8	4	0.9	+	0	0

* infectivity in dex/0.02 ml.

** reciprocal of antigen dilution giving at least a 3+ fixation with 1:8 dilution of Orungo MAF.

*** positive CF reaction with undiluted antigen.

TABLE 12b

DEVELOPMENT OF VIRUS INFECTIVITY AND CF ANTIGEN IN DIFFERENT SECTIONS OF THE BRAIN OF 2-3-DAY OLD SWISS ALBINO MICE INOCULATED WITH 1000 LD_{50} OF ORUNGO VIRUS

Days Post Infection	WHOLE BRAIN		LEFT CEREBRUM		RIGHT CEREBRUM		CEREBELUM		SPINAL CORD	
	Infectivity	CF	Infectivity	CF	Infectivity	CF	Infectivity	CF	Infectivity	CF
1	2.2*	2**	1.5	0	0.8	0	0.9	0	0	0
2	3.8	8	2.3	2	2.1	4	2.3	2	0.8	0
3	4.2	16	3.6	8	3.6	8	3.3	8	1.9	+***
4	6.0	32	5.7	16	5.2	16	4.2	16	3.0	4

* infectivity in dex/0.02 ml.

** reciprocal of antigen dilution giving at least a 3+ fixation with 1:8 dilution of Orungo MAF.

*** positive CF reaction with undiluted antigen.

The half-mouse fixed in Rossmann's fluid was processed in a routine manner in an automatic tissue processor. The sections of the paraffin-embeded blocks were cut with a rotary microtome and stained with hematoxylin and eosin.

Isolated organs were treated in similar manner as whole mice. When 10 or 21-day old whole mice were used for histopathologic studies, they were decalcified prior to treatment in an automatic tissue processor.

For direct immunofluorescence, hyperimmune MAF to Orungo virus was fractionated by DEAE-sephadex chromatography. The immunoglobulin fractions were conjugated with fluorescein isothiocyanate (FITC) according to the method of Murphy et.al. (1973). For indirect immunofluorescence hyperimmune anti Orungo virus - MAF and FITC - goat anti mouse Ig G were used at predetermined dilutions. Substrates included infected and control suckling mouse brain impressions and frozen sections of whole suckling mice. Specimens were examined in a Zeiss microscope equipped with a UG-1/41 filter system and mercury arc light source. Controls which in all cases were negative included adsorption, inhibition and uninfected substrate tests.

Histopathologic changes in moribund newborn mice were restricted to the brain. There was diffuse mononuclear cell infiltration, prominent perivascular and interstitial edema, necrosis of the parenchyma of the cerebrum and also of the granular layer of the

cerebellum (Plates 7, 8 & 9). Ten-day old mice showed only mild and focal mononuclear cell infiltration with perivascular cuffing; "rod cell" formation, that is, elongation of microglia cells (Plate 10). These pathologic changes were restricted to the cerebral cortex. The 21-day old mice were histologically unremarkable.

Immunofluorescent stainings of sections of moribund whole mouse showed widespread foci of viral antigens in the cytoplasm of large neurons (Plate 11). Antigens occured in large aggregated masses. No extra-neural fluorescence was detected.

4.5.2 Experimental infection of golding hamsters (Mesocricetus auratus) with Orungo virus

Newborn (2-day-old) and 3-week-old hamsters were inoculated either IC, IP or SC with 10 mouse LD_{50} or 100 mouse LD_{50} of Orungo virus. Daily, for five days or longer, blood and brain samples were assayed for virus content in suckling mouse. The daily samples of hamster brain were also examined for CF antigen. The presence of Orungo virus in brains of sick baby mice subinoculated with hamsters blood suspension was also checked by CF test.

No hamster was killed following inoculation of Orungo virus by the SC route. Three-week old hamsters survived inoculation with either 10 LD_{50} or 100 LD_{50} of Orungo virus by IP route. Of 24

Plate 7

Deep cerebrum of moribund suckling mouse infected with Orungo virus, showing necrosis, karyorrhexis, perivascular cuffing, interstitial edema and diffuse inflammatory cellular infiltration. X277.

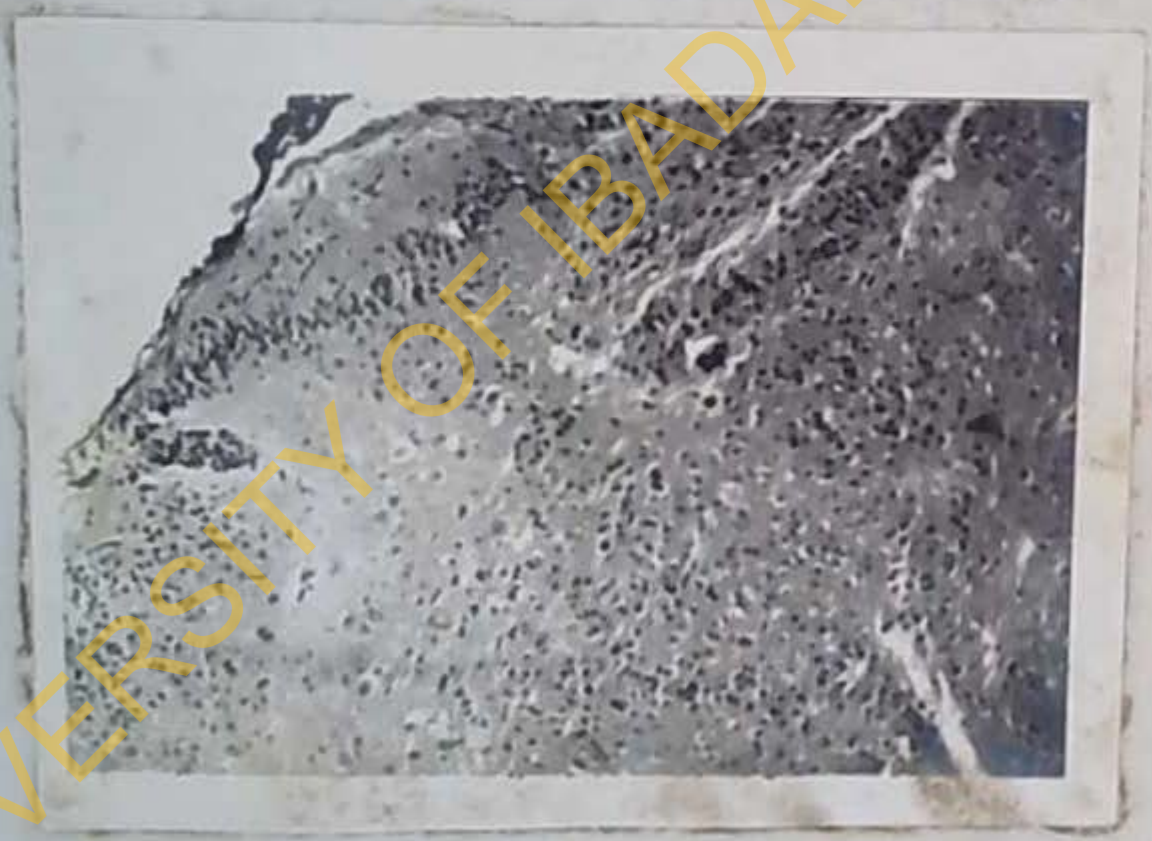
Plate 8

Cerebral cortex of moribund suckling mouse. Necrosis and edema in cerebral nuclear layers. X445.

Plate 9

Cerebellum of moribund suckling mouse. Marked infiltration of inflammatory mononuclear cells, edema and necrosis in the granular layer. X117.

Plate 10



Cerebral cortex of 10-day old moribund mouse showing mild focal mononuclear infiltration. X100.

Plate 11

Focal distribution of Orungo virus specific immunofluorescence in the cytoplasm of neurons. Foci such as this were present randomly in the grey matter of the brain.

X400.

two-day old hamsters inoculated by the same route, with 10 LD_{50} twelve survived; those with evidence of infection died on day 11, 12 and 13 p.i. Twenty of 24 hamsters inoculated by the IP route with 100 LD_{50} of virus, succumbed to infection, death occuring on day 9, 13 and 17 p.i. Viremia was detected from day 2 to day 4 p.i. with 10 mouse LD_{50} infecting does, and day 2 p.i. with 100 mouse LD_{50} infecting dose. Brains of baby hamsters which succumbed to infection by the IP route were harvested and titrated in mice. Virus titres ranged between 4.2 dex and 5.0 dex.

All the hamsters inoculated with Orungo virus by the IC route succumbed to infection with an AST of 4.0 days and 3.5 days for 10 LD_{50} and 100 LD_{50} of infective virus doses respectively. There was viremia between day 3 and day 5 p.i. The peak of viremia coincided with the peak virus titre in the brain (Table 13). The development of infectious virus and complement fixing antigen in baby hamsters following IC inoculation is shown in Fig. 15. There was a steady increase in the amount of the virus in the brain. Baby hamsters which survived IP or SC inoculation with Orungo virus, as well as adults inoculated by the SC or IP routes were bled out 30 days post infection. Sera from each group of animals were checked by N test for Orungo virus antibody. Sera from 2-day old hamsters inoculated by the IP route neutralized on the average 1.6 dex (range 1.1 dex - 1.8 dex) of Orungo virus; 3 week old

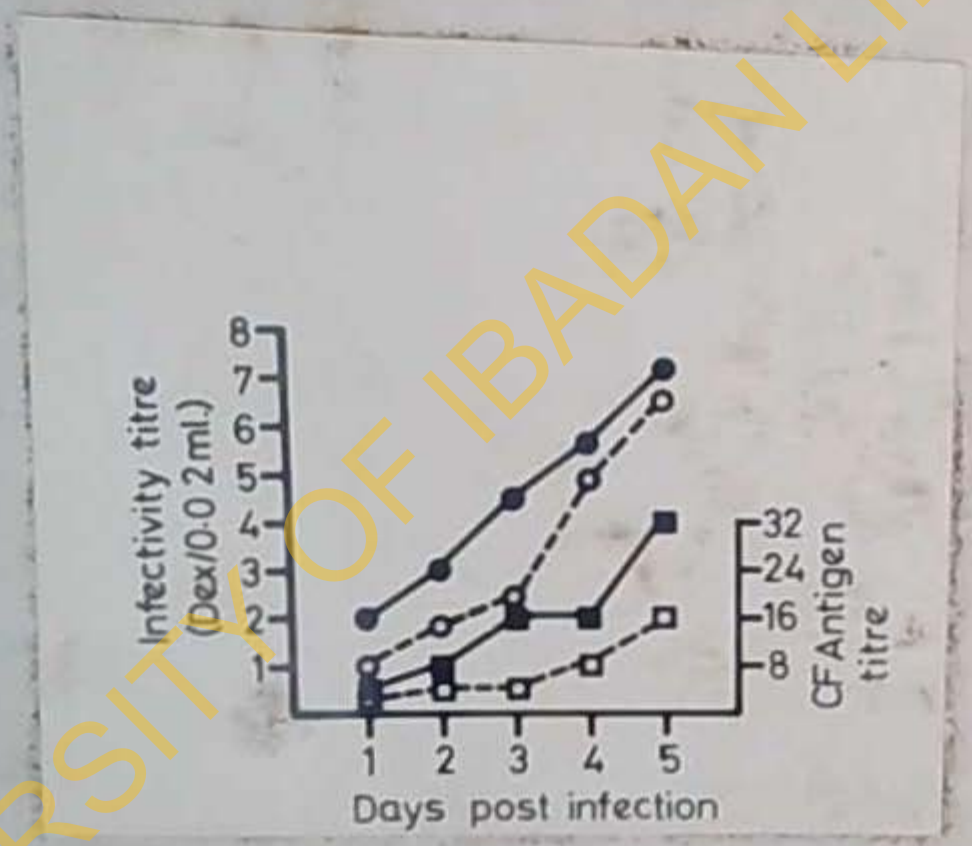
TABLE 13

DEVELOPMENT OF INFECTIOUS VIRUS IN THE BRAIN AND PATTERN OF VIREMIA IN HAMSTERS INOCULATED IC WITH ORUNGO VIRUS

Age	Route	Dose in Mouse LD_{50}	AST in days	Infectivity titres in Dex/ 0.02ml. BLOOD Day Post Infection					Infectivity titres in Dex/ 0.02ml. BRAIN Day Post Infection				
				1	2	3	4	5	1	2	3	4	5
2 days	IC	10	4.0	0	0	0	0.6	1.6	0.6	1.7	2.4	4.8	6.5
2 days	IC	100	3.5	0	0	1.2	0.8	1.2	2.0	3.0	4.5	5.6	7.2
2 days	IP	10	14.0	0	0	0.6	1.5	0	NT	NT	NT	NT	NT
2 days	IP	100	14.0	0	1.2	0	0	0	NT	NT	NT	NT	NT

NT = not tested, however virus titre range in brains of hamsters dying between day 9 p.i through day 17 p.i. was 4.2 dex - 5.0 dex.

Fig. 15



Development of Orungo virus infectivity and complement-fixing antigen in hamster brain; (100 LD_{50}: closed circles = infectivity, closed squares = CF. 10 LD_{50}: open circles = infectivity, open squares = CF).

TABLE 14

RESULTS OF NEUTRALISING ANTIBODY STUDIES IN SERA OF HAMSTERS INFECTED WITH ORUNGO VIRUS

Age	Route of Infection	Log of Neutralisation Index*			
		Group 1	Group 2	Group 3	Average
2 days	IP	1.2	1.8	1.7	1.6
2 days	SC	1.9	2.2	1.8	2.0
3 weeks	IP	2.5	2.9	-	2.7
3 weeks	SC	2.4	2.6	-	2.5

* LNI expressed as dex

hamsters inoculated by the same route, neutralized 2.7 dex of Orungo virus (Table 14). By the SC route 2-day and 3-week hamsters neutralized on the average 2.0 dex and 2.5 dex of Orungo virus respectively.

4.5.3 Experimental infection of domestic rabbits, Oryctolagus cuniculus, with Orungo virus

Ten rabbits, 8-10 weeks old, were used for this experiment. The animals were kept two per cage and observed for ten days prior to commencement of the experiment. Four routes of inoculation, intravenous (IV), intraperitoneal (IP), subcutaneous (SC), and conjuctival sac, were used. The routes and virus doses employed are shown in Table 15. Animals were bled for the first seven days post inoculations, and the blood samples assayed for virus content, by IC inoculation of baby mice. At pre-determined intervals both before and after infection 5 cc of blood was collected for serology. Sera were checked in neutralization test for specific Orungo virus antibody.

No virus was isolated from any of the blood sample collections. All rabbits except those infected by the SC route, developed specific N antibodies to Orungo virus (Table 15). The time of development and the level of antibody was dependent on the amount of virus inoculated. By the IV route, Orungo virus N-antibody developed faster and reached a higher level when infectivity

TABLE 15

DEVELOPMENT OF NEUTRALISING ANTIBODIES IN RABBITS FOLLOWING INOCULATION WITH ORUNGO VIRUS BY DIFFERENT ROUTES

Animal No.	Route	Dose in Dex/ml	LOG OF NEUTRALISATION INDEX* -7** days	+14 days	+28 days	+42 days	+94 days
893	IV	4.0	0	0	0.8	0.7	0.6
895	IV	8.0	0	0.6	2.0	2.0	2.3
905	IP	4.5	0	0	0	0	0.8
938	IP	8.2	0	1.2	2.0	2.0	3.8
908	SC	6.9	0	0	0	0	NT
916	SC	7.2	0	0	0.3	0	0
893	Conjuctival	5.0	0.0	1.0	1.9	NT	1.8
907	Conjuctival	6.5	0	0.9	1.5	1.7	2.0
917	Control	-	0	0	0	0	0
918	Control	-	0	0	0	0	0

* expressed in dex.

** days post infection.

virus dose was 8.0 dex as compared with a virus infecting dose of 4.0 dex. Similar results were obtained following IP infection. Significant neutralizing antibodies persisted in these animals up till 94 days p.i. when the experiment was terminated.

4.5.4 Experimental infection of lambs with Orungo virus

Six lambs (4-6 weeks old) cross of ewes and rams maintained in the Virus Research Laboratory, were used for this experiment. Animals were inoculated with either 3.0 dex or 5.0 dex of Orungo virus by SC or IV route. They were then bled for the first seven days post inoculation and the blood samples assayed for virus content. At pre-determined intervals, 5-10 cc of blood was collected for serology. Sera were tested for Orungo virus N-antibody in suckling mice. No virus was isolated from any of the blood samples collected. Only animals infected by the IV route, developed N antibodies to Orungo virus. The time of development of antibody and the level of antibody was a factor of developed virus dose. At the higher virus dose N antibody reached a higher level. Peak antibody titre was detected at 28 day p.i., and no antibody was detected by day 63 p.i. (Table 16).

4.5.5 Experimental infection of house sparrows (Passer domesticus) with Orungo virus

Thirty adult house sparrows were infected with 3 dex of Orungo virus contained in 0.1 ml. Ten each were infected by the IV, SC

TABLE 16

ANTIBODY RESPONSE IN LAMBS FOLLOWING INFECTION WITH ORUNGO VIRUS

Animal No.	Route of Infection	Dose in Dex/ml	LOG OF NEUTRALISATION INDEX (in Dex/0.02 ml.)					
			-5 pi	+14 pi	+28 pi	+42 pi	+56 pi	+63 pi
441	IV	5.0	0	0.8	2.0	1.8	0.5	0
442	IV	3.0	0	0	0.5	0.6	0.2	0
443	SC	5.0	0	0	0	0	0	0
444	SC	3.0	0	0	0	0	0	0
445	Control	0	0	0	0	0	0	0
446	Control	0	0	0	0	0	0	0

and oral routes. Viremia studies were carried out for each day of the first seven days following infection. On day 14 and day 28 p.i., five animals from each group were bled out and the sera titrated in baby mice for presence of N antibody to Orungo virus.

House sparrows neither circulated Orungo virus, nor developed (N) antibody to the virus following infection by the IV, SC and Oran routes.

4.5.6 Experimental infection of baby chicks with Orungo virus

Two groups of one-day-old chicks were used for these experiments. Each of the six chicks in the first group was inoculated with 0.1 cc of 10^{-2} dilution of Orungo virus (equivalent to 100 LD_{50}), by the IV route. The other group was inoculated with the same dose of virus by the SC route. Daily, bleedings were done for the first seven days following inoculation, and the samples tested for presence of virus. Specific Orungo virus antibody were assayed for in neutralization tests in sera samples collected on day 14 and day 28 p.i.

As was the case with house sparrows day-old chicks failed to circulate virus, nor did they develop specific (N) antibodies to Orungo virus.

4.6 Studies on the transmission of Orungo virus by Aedes albopictus and Aedes aegypti mosquitoes

The Ib AR.52302 strain of Orungo virus isolated from a pool of Aedes dentatus mosquitoes in Nigeria was used for these studies. The strain was at the seventh mouse brain and second BHK-21 tissue culture passage level. Virus stock titre was $10^{-4.0}$ TCID50/ml.

Two species of mosquitoes Aedes albopictus and Aedes aegypti were used. The Aedes albopictus mosquitoes came originally from Poona in India; and the Aedes aegypti came from Amhur strain in Thailand. Both species were maintained in the entomology laboratory of the Yale Arbovirus Research Unit, in New Haven, U.S.A. where these studies were conducted.

From experience there was no known laboratory hosts which showed high level of viremia when infected with Orungo virus. Indeed, infection by the SC route produced no viremia at all in four experimental animals so inoculated. Infection of the mosquitoes was therefore accomplished either by intra-thoracic (IT) inoculation of mosquitoes with virus suspension, or by permitting them to engorged on virus chick blood suspension through an egg shell membrane or cotton pledgets.

Intrathoracic inoculation: The inoculating needle was made out of a melting point capillary tubing drawn into a sharp point over a hot flame. The tip was broken at a selected point with a

pair of jeweller's forceps under a dissecting microscope and marked off in 1 mm graduations. Inoculation of undiluted virus stock was through the membrane just anterior to the sterno-pleuron and below the first thoracic spiracle. Each mosquito was inoculated with .0006 ml of virus suspension (1 mm on the needle). All manipulations were carried out on adult female mosquitoes lightly anesthesized with carbon dioxide and immobilized on wet ice.

Engorgement on virus blood meal: Adult female mosquitoes (starved for 24 hours prior to feeding on virus-blood meal) were placed in holding cages and allowed to feed on cotton pledgets soaked in the virus-blood meal, or through an egg-shell membrane. The egg shell contained 1 ml of the virus-blood meal. The virus blood meal was made up of 2 ml of undiluted virus stock, 7.5 ml of inactivated and defibrinated chick blood and 0.5 ml of antibiotic solution.

Inoculated and engorged mosquitoes were then placed in bobbinet covered, cylindrical plastic cages and provided with maintenance diet of 10% dextrose through cotton pledgets. The mosquitoes were held for 6 to 11 days at 26°C and 68%-78% relative humidity. Immediately following mosquito inoculations (2 hrs. or less after the start of these operations), the virus blood meal was titrated in mice.

Transmission attempts: At intervals, following exposure to infection, mosquitoes were allowed to bite 1-2 day old mice.

Engorged mosquitoes were stored at -70°C until tested for virus by grinding and inoculating the mosquito material into baby mice. Bitten mice were watched for signs of illness and virus isolations made from brains where indicated. Brain suspensions (10% in BAPS) of sick mice were passaged IC to groups of fresh mice until a typical infection resulted. Brain suspension from one or more of mice showing the typical infection was then set up in a CF test to establish virus identity.

Two other methods were employed to show possible transmission of Orungo virus by mosquitoes. In the first method mosquitoes were allowed to engorge on a drop of blood meal (equal parts of 2.5% fetal calf serum (FCS), 10% dextrose and inactivated, defribrinated chick blood). One mosquito was placed into a tube stoppered at one end with cotton plug and covered at the other with synthetic fine mesh netting material. A drop of the blood was placed on the netting material and the mosquitoes allowed to engorge. For the second method, the mosquitoes engorged from a blood meal in a capillary tube. This method has been fully described by Aitken, (1975). Briefly, the mosquito was slightly anesthesized with CO_2 fumes, and the first two pairs of legs removed under a dissecting microscope. The proboscis was next pushed through the narrower end of an inoculating needle, which was previously filled with the blood meal to a marked level. The mosquitoes that engorged on

the blood meal through any of the method - feeding on susceptible mouse, blood droplet or capillary feeding, were tested for virus by grinding and inoculating the mosquito material into baby mice. The inoculated mice were watched daily for signs of illness and subsequently, procedures for the isolation and identification of virus causing illness were carried out.

Each of the blood meal on which the mosquitoes have engorged (blood drop, or capillary) was then mixed with a drop (about 0.03 ml) of 2.2% FCS and inoculated intrathoracically into 10-15 adult Aedes albopictus or Aedes aegypti females. These were kept for 7-10 days at 26°C and 68%-78% relative humidity. They were maintained on 10% dextrose. At the end of the incubation period, all dead mosquitoes were removed and remaining tested for virus by i.c. inoculation of 2-day old baby mice. Brain materials from sick mice showing typical infection were harvested and tested for virus. The results of the transmission of Orungo by Aedes albopictus and Aedes aegypti mosquitoes are presented in Table 17.

Aedes albopictus: Following parenteral inoculation of 1.8 dex of Orungo virus per mosquito, artificial transmission was achieved by allowing mosquitoes to "bite" on blood droplets on day 6 and day 10 of extrinsic incubation. Three of six mosquitoes transmitted Orungo virus on day 6, although only one of the six was positive for virus with a titre of 2.3 dex. The level of virus injected

TABLE 17

TRANSMISSION OF ORUNGO VIRUS BY BITE OF INOCULATED MOSQUITOES

Routes of Infection	Species of Mosquitoes	Days of Extrinsic Incubation	Number of mosquitoes positive for virus		Number of mosquitoes transmitting virus	
			Number Tested		Number of Transmission Attempts	
			Mice	Blood Droplet	Mice	Blood Droplet
INTRATHO-RACIC	Aedes albopictus	6	1/5	3/6	0/5	3/6
		7	0/2	1/9	0/2	0/9
		10	2/7	1/4	0/7	1/4
			3/14	5/19	0/14	4/19
ORAL	Aedes albopictus	6	1/3	0/5	0/3	0/5
		7	0/2	0/8	0/2	0/8
		11	-	0/7	-	0/7
			1/5	0/20	0/5	0/20
INTRATHO-RACIC	Aedes aegypti	7	-	1/3	-	1/3
			-	1/3	-	1/3
ORAL	Aedes aegypti	7	-	0/2	-	0/2
		9	-	0/3	-	0/3
			-	0/5	-	0/5

into the blood droplets by the other two mosquitoes transmitting virus required further passage in fresh mosquitoes before subsequent isolation and identification. On the same day, none of the 5 mosquitoes which fed on susceptible mice transmitted virus to the host. Only one of these mosquitoes was infected, with a virus titre of 2.8 dex. On day 7, nine mosquitoes engorged on blood droplets, and none transmitted virus, although only one was positive for virus (titre 2.2 dex). The two inoculated mosquitoes feeding on mice were negative for virus and failed to transmit same to bitten mice. On day 10, one mosquito with a virus titre of 3.0 dex "transmitted" virus to a drop of blood. The other three were negative for virus and did not transmit virus. Of the 7 mosquitoes that fed on mice, two of which were infected, (virus titre 2.4 dex and 2.7 dex) none transmitted virus to the mice. One mosquito, of those that engorged on the blood meal containing virus, had a titre of 1.9 dex, however it failed to transmit virus to susceptible mouse after six days of extrinsic incubation. No virus was found in the other six, which also did not transmit virus to susceptible mice on which they fed.

Aedes aegypti: On day 7 of extrinsic incubation following a parenteral inoculation of Aedes aegypti mosquitoes with 1.8 dex of Orungo virus per mosquito, one of the three tested, transmitted the virus. It had a titre of 2.5 dex. None of the five Aedes

aegypti mosquitoes which engorged on virus-blood meal contained any virus, nor did they transmit virus. They were tested on day 7 and day 9 of extrinsic incubation.

4.7 Tissue culture susceptibility studies

Five strains of Orungo virus were tested for their ability to propagate and multiply in Vero, BHK-21, and Aedes albopictus cell lines. Details of establishment and maintenance of each of the cell lines have been given under materials and methods (section 3.7).

Ten percent mouse brain suspensions prepared in maintenance medium (MEME) supplemented with 2% inactivated FCS, were inoculated onto confluent cell culture monolayers from which medium had been drained off. Each tube was inoculated with 0.1 ml of virus suspension. After viral adsorption at 37°C for 1 hr. the virus suspension was drained off and the monolayer washed with Hank's BSS (containing no FCS). Fresh maintenance medium was then added. The tubes or bottles were incubated at 37°C and observed daily for CPE.

4.7.1 Orungo virus in Vero cell cultures - Cercopithecus monkey kidney line

Following standard procedures and after six blind passages, no CPE was demonstrated on infection of Vero cell cultures with

Orungo virus. A slight modification to the standard procedures was then used: At 24 hourly intervals, infected and control cells were trypsinized using a 0.25% solution of typsin in Hank's balanced salt solution. The dispersed cells were regrown by subculturing into fresh bottles containing no cells and reincubated at 37°C with daily examinations for CPE. With this modification, Orungo virus was adapted and propagated in Vero cell cultures. From preliminary observations, trypsination between 24-48 hours post infection, before overgrowth and aging of cells consistently resulted in production of CPE. The trypsinization and subculturing of infected cells was only carried out at the initial stage of infecting cell cultures with mouse brain suspensions. With the production of CPE following this initial trypsinization, it was possible to infect fresh confluent cells using culture fluids and produce CPE without trypsinization. The time of appearance of CPE decreased with increasing passages until the 5th passage (Table 18), when CPE appeared regularly from day 2 post infection. Complete CPE was achieved by day 3 or 4 post infection. Only the result of Ib H13019 strain of Orungo virus are presented as the other four strains gave similar results.

4.7.1.1 Rate of replication of Orungo virus in Vero cell cultures 37°C and room temperature

The growth rate of Ib H.13019 strain of Orungo virus was performed in Vero in cell monolayer cultures grown in tubes.

TABLE 18

ADAPTATION AND PROPAGATION OF ORUNGO (H13019) VIRUS IN VERO CELLS FOLLOWING TRYPSINISATION AND SUB-CULTURING

PASSAGE NO.	CPE	TITRE		CFT	
		DEX/0.02 ml. IN MOUSE	DEX/0.02 ml IN VERO CELL	MOUSE BRAIN	TC FLUID
1	+(4)*	NT**	NT	NT	NT
2	+(4)	NT	NT	0	0
3	+(3)	0.6	NT	0	0
4	+(3)	NT	NT	NT	NT
5	+(2)	1.1	1.0	+	2***
6	+(2)	NT	NT	+	2
7	+(2)	NT	2.1	+	4
8	+(2)	3.4	2.5	+	8
9	+(2)	NT	3.1	+	4
10	+(2)	4.5	3.7	+	4

* day post inoculation on which CPE first appeared.

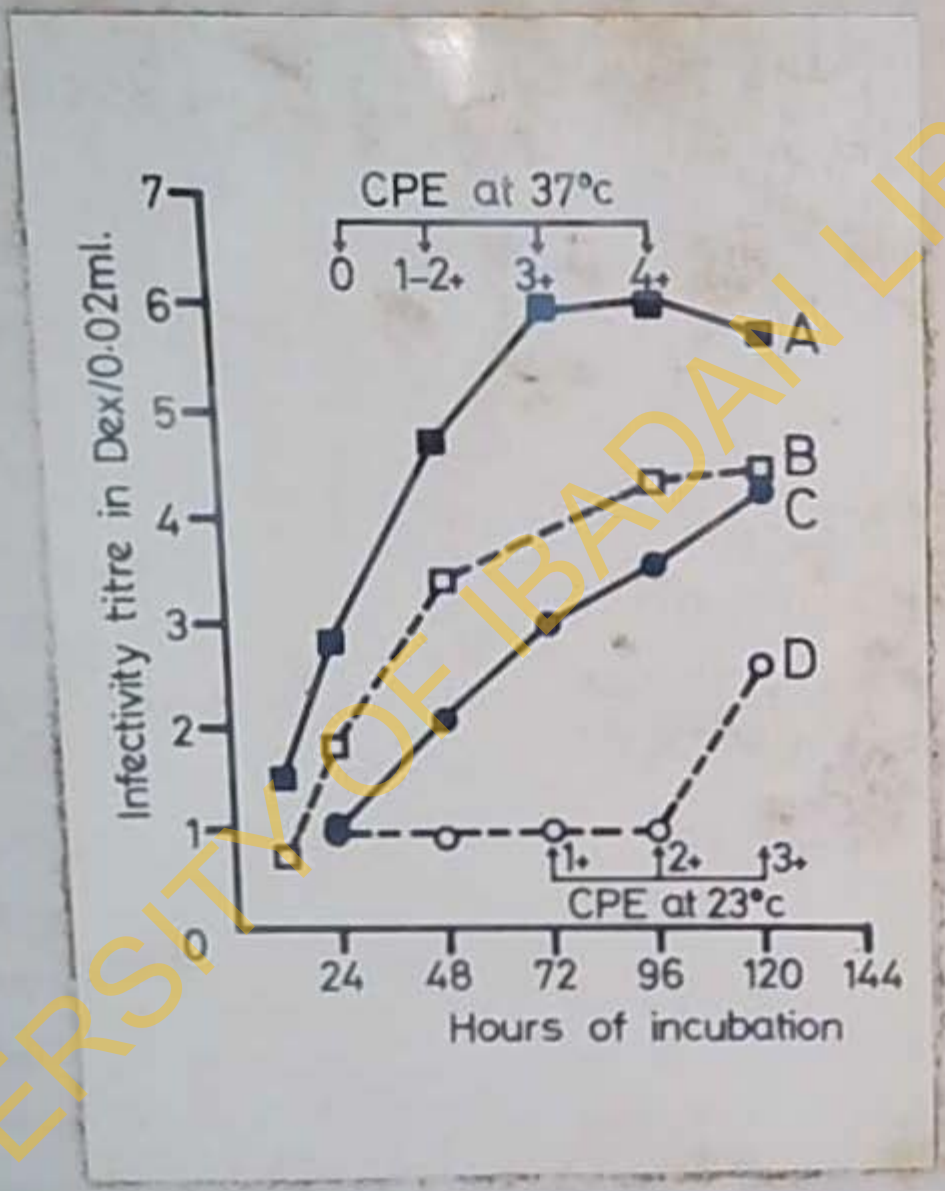
** NT not tested.

*** CF titre, reciprocal of highest dilution of culture fluid giving a 3+ fixation.

Confluent monolayers were washed three times with Hank's BSS and each tube was inoculated with 0.1 ml of virus suspension containing 5.0 dex $TCID_{50}$/ml in MEME supplemented with 5% FCS. Virus adsorption was allowed to take place for one hour, at 37°C or room temperature (23 ± 1°C) after which, monolayers were washed three times with Hank's BSS. Each tube was then fed with 1.0 ml of MEME supplemented with 2% FCS, and reincubated at 37°C or 23°C. At 6, 12, 24, 48, 72, 96 and 120 hr. post infection five tubes were harvested and treated as follows: The fluids from each of the tubes were pooled, and the monolayer culture rinsed with Hank's BSS. Cells were scraped off each tube with a rubber policeman. The scraped cells were then suspended in the 1.0 ml of medium. The pooled fluids and cell suspensions were stored at -70°C until titrated in baby mice.

The replication of Ib H.13019 strain of Orungo virus in Vero cells in respect of the time of incubation and cell— and fluid-associated virus is shown in Fig. 16. Cultures were inoculated with 4.0 dex of Orungo virus. At 37°C and 6 hours of incubation, 1.0 dex of infectious virus was detected in the cells, while no virus was detected in the fluids. At 12 hours, virus replication was first detected with a titre increase to 1.5 dex in the cells. Replication increased until cell-associated virus reached a peak titre of 6.0 dex between 72 and 96 hours of

Fig. 16



Replication of Orungo (Ib.H13019) virus in Vero cells at 37°C (A = cell associated, B = fluid associated virus) and 23°C (C = cell associated, D = fluid associated virus).

incubation. Virus titre in the fluid increased and reached a peak of 4.5 dex at 120 hours post incubation. CPE was first detected between 24-48 hours post incubation, complete CPE coincided with peak cell-associated virus titre.

At 23°C, virus replication was first detected at 48 hours of incubation in cells, and peak titre was 4.3 dex after 120 hours of incubation. No virus was detected in the fluids, until between the 96th and 120th hour of incubation when there was a dramatic increase in virus titre from below 1.0 dex to 2.5 dex. CPE was first observed at 72 hours post incubation, but was not complete at 120 hours of incubation when the experiment was terminated.

Cellular morphological alterations following virus infection was also studied in Vero cells. Vero cell cultures were prepared in Leighton tubes with coverslips as described by Malmquist (1962). When cells were confluent, the fluid in the tube was replaced with maintenance medium and the cell cultures inoculated with 2 dex $TCID_{50}$ of Orungo virus. Control uninoculated tubes were set up in parallel and coverslips from both inoculated and control tubes were removed for staining as follows: Fixation of cells was carried out in full strength methyl alcohol for 20 minutes and left to dry at room temperature. The coverslips were then stained with 10% Giemsa dye at room temperature for between ½ to 1 hour and washed in distilled water.

A normal, uninfected confluent Vero cell monolayer culture is shown in Plate 12. Early (2 days post incubation) and late (3-4 days post incubation) CPE of Orungo virus in the same cell are shown in Plates 13 and 14. Early stages CPE was characterized by darkly staining homogenous nuclei with loss of nucleoli and loss of detailed nuclear internal structure. Cellular outline began to disappear with a few pockets of clear spaces in the cell sheet, evidence of cellular necrosis. Late stage CPE showed a greater degree of cellular necrosis with aggregation of darkly staining nuclei due to complete degeneration of cytoplasmic material.

4.7.2 Orungo virus in BHK-21 cell cultures

BHK-21 cell cultures were grown at 36°C in MEME supplemented with 10% calf serum, they were maintained on the same medium with 2% calf serum. Cells were grown in bottles for passages, in tubes for growth curve, and in Lab-Tek slide chambers (8 wells/slide, Miles Laboratories, Napperville, Illinois) for immunofluorescence and histology. Serial harvests terminating at complete CPE were made for growth curve, immunofluorescence and histology.

Immunofluorescence staining was as previously described for mouse brain studies, using infected and control BHK-21 cells as substrates. For histology, infected and control culture cells were stained by the Giemsa method.

Plate 12

Normal monolayer of Vero cells. X250.

Plate 13

Monolayer of Vero cells showing cytopathic effect of Orungo virus 2 days post incubation. X250.

Plate 14

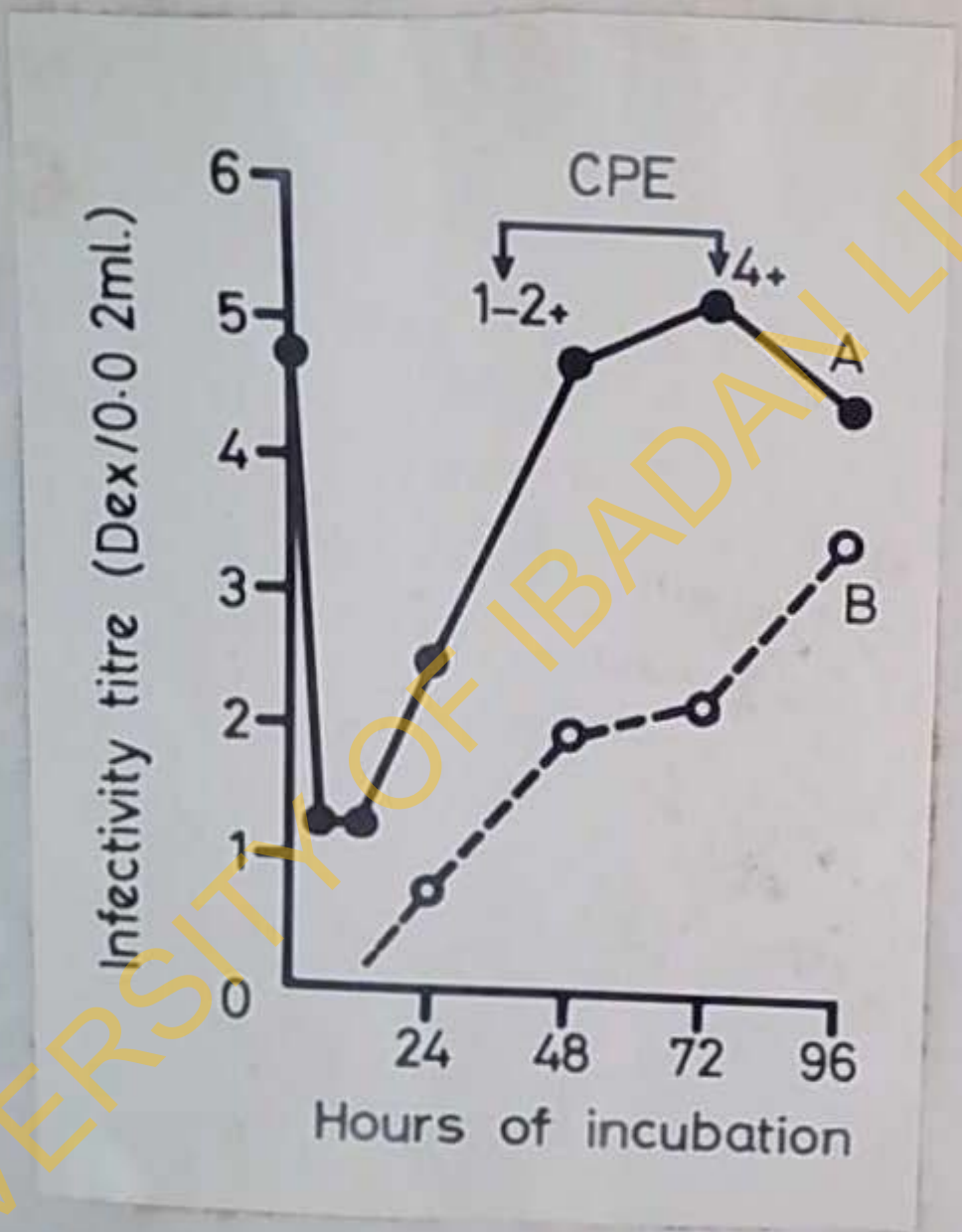
Monolayer of Vero cells showing cytopathic effect of Orungo virus 3-4 days post incubation. X250.

The rate of growth of Orungo (Ib H.13019) virus in BHK-21 cells is shown in Fig. 17. Cultures were inoculated with 4.7 dex of Orungo virus. At 6 hours as well as 12 hours incubation, 1.2 dex of virus was detected in the cells while the fluid was negative for virus. Virus replication was first detected at 24 hours incubation, with a titre increase to 2.5 dex in the cells and 0.7 dex in the fluids. The peak virus replication was 5.2 dex for the cells at 72 hours incubation. Virus titre peak of 3.5 dex in the fluids was at 96 hours incubation, by which time the cell-associated virus titre had decreased to 4.5 dex, possibly due to release of virus into the fluid medium.

The time of initial appearance of CPE was on day 4, however this decreased with increasing BHK-21 cell passages and stabilised at 36 hours from the third passage onwards. Complete CPE was achieved at 60-72 hours of incubation at 36°C.

The character of the CPE in infected BHK-21 cells as studied by Giemsa staining of sequential harvests, revealed focal rounding of cells with a retraction of cytoplasm resulting in extreme density (Plate 15). This progressed to involve massive aggregates of cells with fusion and necrosis ending in homogenous, basophilic opaque masses (Plate 16). These aggregates remained attached for long periods; thereafter large clear spaces appeared in the cell monolayer, as these masses of necrotic debris detached from the glass (Plate 17). Parallel immunofluorescence microscopy revealed

Fig. 17



Replication of Orungo (Ib H13019) virus in BHK-21 cells at 37°C.

Plate 15

BHK-21 cells infected with Orungo virus showing early cytopathic effect (12 hours). X177.

Plate 16

BHK-21 cells infected with Orungo virus illustrating late stage cytopathic effect (60 hours). X177.

Plate 17

BHK-21 cells infected with Orungo virus showing clear spaces in monolayer where masses of necrotic cellular debris have detached. X177.

a focal distribution of intracytoplasmic antigen at 12 hours in a few cells; by 48 hours the majority of cells showed intracytoplasmic antigen distributed in large punctate masses (Plate 16). Of note was the tendency of dead or dying cells to exhibit bright, specific, fluorescence uniformly in cytoplasm and nucleus; it was concluded that this represented a stage of cytonecrosis when nuclear membrane integrity was lost and antigen was free to fill the still intact cells.

4.7.3 Orungo virus in Aedes albopictus cells line

Aedes albopictus cell line provided by Dr. Sonja Buckley, Yale Arbovirus Research Unit, New Haven, Connecticut, U.S.A., was used for these studies. The cells were grown in 2 oz flint glass bottles and maintained in 80% Mitsuhashi-Maramorosch (M-M) medium supplemented with 20% FCS, (M-M-80-20). Confluent cells were inoculated with 0.2 ml of 5.0 dex of Orungo virus, incubated for 1 hour at room temperature before rinsing with Rinaldini's salt solution. Each bottle was then fed with 5 mls of M-M-80-20 medium. Previous passage history of the Orungo (AR.52302) virus strain used was 7th mouse brain passage and 2nd BHK-21 cell passage. At predetermined intervals fluids and cells were harvested and titrated immediately by CF, mouse inoculation, or inoculation of BHK-21 cell monolayer. Two drops of undiluted from infected cell culture (an

Plate 18

BHK-21 cells infected with Orungo virus. Specific immunofluorescence at 48 hours. X250.

antigen) were reacted against two-fold dilutions of specific Orungo MAF in a routine CF test. Orungo virus failed to replicate in Aedes albopictus line after three successive passages.

A summary of host susceptibility of Orungo virus is presented in Table 19.

4.8 Plaque formation in Vero and BHK-21 cell cultures

The development of plaques in Vero and BHK-21 cell cultures by Orungo virus strains was investigated. One ounce flint glass prescription bottles were seeded with 5 mls of cell suspension containing 150,000 cells/ml and incubated at 37°C for 2 days to obtain confluent sheets. For inoculation of virus, the fluid medium was removed from the cultures and 0.1 ml of each virus dilution was adsorbed for 1 hour at 37°C. This was followed by the addition of 5 mls of freshly prepared nutrient agar overlay containing neutral red, or 3 mls of the overlay without neutral red. Overlaid cultures were incubated in an inverted position at 37°C and examined daily for appearance of plaques. Forty-eight hours later 3 ml of a second overlay containing neutral red was applied to the bottles containing initial overlay medium without neutral red.

All the five Orungo virus strains developed plaques in Vero cells only. There was no difference in plaque size. Plate 19 shows a typical Orungo virus plaque. The difference in time of appearance

TABLE 19

SUSCEPTIBILITY OF VERTEBRATES, ARTHROPODS AND CELL CULTURES TO EXPERIMENTAL INFECTION WITH ORUNGO VIRUS

Experimental Host	Passage history of strain used	Age of mice	Inoculation		Evidence of infection	Average survival time MST (days)	Titre in (dex/0.02 ml)
			Route	ml.			
Mice	7th suckling mouse brain (smb)	1-4 days	ic	.02	Death, viremia	3-4	8.5
		1-4 days	ip	.03	-	-	
		1-4 days	sc	.03	-	-	
		10 days	ic	.02	Death	12-15a	3.2
						6-7b	3.0
			sc	.03	-		
		3-4 week	ic	.03	-		
			ip	.10	-		
Hamsters	7th (smb)	2 days	ic	.02	Death, viremia	3.5-4.0	6.5-7.2
			ip	.03	Death, antibody development	14.0	4.2-5.0
			sc				
		3 week	ip	.10	antibody	-	-
			sc	.10	antibody		
Rabbits	3rd (smb)	10 weeks	con.*	.10			
			ip	2 & 10	Antibody in 3-4 weeks p.i.		
			iv	2			
			sc	2 and 5			
Sheep	3rd (smb)	4-6 weeks	iv	1.0	Antibody		
			sc	1.0	-		

*conjuctival route.

TABLE 19 (continued)

Experimental Host	Passage history of strain used	Age of mice	Inoculation Route	Inoculation ml.	Evidence of infection	Average survival time AST (days)	Titre in (dex/0.02 ml)
Sparrows	7th (smb)	Adult	iv	0.1	-		
			sc	0.1	-		
			oral	0.1	-		
Chicks	7th (smb)	1 day old	iv	0.1	-		
			sc	0.1	-		
Cell culture:							
BHK-21	7th (smb)				CPE	2-3	5.2
Vero	"				CPE, plaques	2-3	6.0
Aedes albopictus line	"				No multiplication	-	-
Aedes albopictus	7th (smb)	Adult	Intra-thoracic	.0006	Time 0 2.0 dex/ mosquito. Day 6 2/11 mosquitoes infected with 2.3 dex and 2.8 dex of virus. Day 7 1/11 mosquitoes infected with 2.2 dex. Day 10 3/11 mosquitoes infected with 2.4 dex, 2.7 dex and 3.0 dex of virus respectively.		Transmission positive. No transmissio Transmission positive.
Aedes aegypti	7th (smb)	Adult	Intra-thoracic	.0006	Time 0 1.8 dex/mosquito. Day 7 1/5 mosquitoes infected with 2.5 dex of virus.		Transmission positive.

a = infecting virus dose 10 LD_{50}

b = infecting virus dose 100 LD_{50}.

Plate 19

Orungo virus plaques in Vero cells. X1

of the plaques was dependent on the type of overlay medium used. When the double overlay was used plaque appeared within 3 days of incubation; with the single overlay, plaques appeared 48 hours later. In general, the titres of virus strains treated with double overlay were between 10^1 - 10^2 plaques forming units (pfu) greater than those with single overlay.

4.9 Antigenic analyses of Orungo virus

4.9.1 Antigenic relationship of Orungo virus to other viruses

Eight strains of the virus isolated in Nigeria which were earlier reported to be antigenically indistinguishable from the prototype Orungo virus - UgMP 359 strain isolated in Uganda, were screened in CF tests against over 150 viruses representing the major virus groups: Alphaviruses, Bunya and Bunya-like viruses, Flaviviruses, Orbiviruses, Rhabdoviruses and other unclassified viruses. The Uganda prototype virus strain was also included in these tests.

One of the nine strains tested, (H.60974) gave positive CF reactions with Tataguine virus. The other eight strains including the Uganda strain gave negative results with the antigens, or antisera/antiMAPS or both of the following viruses.

Alphaviruses: Chikungunya, Mayaro, Middleburg, M'dumu, O'nyong-nyong, Semliki Forest, Sindbis.

Arenavirus: Lassa, Tacaribe.

Bunyavirus: Akabane, Batai, Bunyamwera, Botambi Buttonwillow, Bwamba, Cache valley, California encephalitis, Capin, Germiston Guama, Ilesha, Ingwavuma, Keystone, La Crosse, Lokern, Lumbo, Maguari, Main Drain, Manzanilla, Marituba, Melao, Olifantsvlei Oropuche, Pongola, Sabo, Tete, Trivittatus, Ukauwa, Utinga, Yaba 7.

Bunyavirus-like: (similar morphology, but serologic dissimilarity to Bunya viruses): Arumowot, Bakau, Bhanja, Chagres, Congo, Dugbe, Ganjam, Itaporanga, Mpoko, Rift Valley, Sandly fever, Tataguine, Thogoto, Turlock, Umbre.

Flaviviruses: Banzi, Bouboui, Bukalasabat, Bussuquara, Dakar bat, dengue 1,2,3,4, Entebbe bat, Ilheus, Koutango, Modoc, Ntaya, Saboya, Spondweni, Tyuleniy, Uganda S, Usutu, Wesselsbron, West Nile, Yellow fever and Zika.

Orbiviruses: African horse sickness, Baku, bluetongue, Chaguinola, Chenuda, Colorado tick fever, epizootic hemorrhagic disease of deer (EHD), Eubenangee, Konorovo, Lebombo, Mono Lake, Palyam, Umatilla, Wad Medani.

Rhabdoviruses: Bovine ephemeral fever, Chandipura, Flanders, Hart Park, Kotonkan, Lagos bat, Mokola Mosqueiril.

Unclassified viruses: Bandia, Boteke, Cossas, Joa, Kouraliba, Le Dantec, Mkolbisson, Nyamanini, Nyando, Okola, Quaranfil, Tanga, Touro, Yogue.

Others: Ib An 10964, Ib An 10069, Ib An 17143, Ib An 33709, Ib H 41795, Ib H 51378, Ib An 54147, Ib An 22619, Herpes, New Castle Disease virus (NDV), Coxsackie virus A & B.

4.9.2 Antigenic similarities between Orungo virus strains

Seven Orungo virus strains isolated in Nigeria and the prototype strain from Uganda were compared by CF tests for possible antigenic differences. Included in this study was strain H60974, which had earlier been reported as a strain of Orungo virus, Monath et al. (op. cit.) but which in several CF tests was found to be related to Tataguine virus and not Orungo virus. Sucrose-acetone extracted antigens produced for each strain according to the method of Clarke and Casals (op. cit.) served as the source of CF antigens. Immune MAFS to each virus strain were produced using one, two or four injection(s), Freund's adjuvant and sarcoma 180 TC cells as described by Tikasingh et al (op. cit.). The MAFS thus produced represented stages of reactivity from specific to broad-reacting. Immune fluids were diluted 1:4 with veronal buffer diluent and inactivated at 56°C for 30 minutes. Two fold serial dilutions of the immune fluids were reacted in a "Checkerboard" or "cross block" CF test with two-fold serial antigen dilutions. The immune fluids were distributed into appropriate wells in plates with a microtitre

dropper which delivered 0.025 ml per drop. One drop of complement containing 2 units was added to each well. The diluted antigens were then distributed according to the plan of the test, one drop per appropriate well. In addition to the viral antigens and DIAPS employed in the test, normal mouse brain and normal mouse fluid were included as controls. Anticomplementary controls, antigen or immune fluids with diluent were also included for each immune fluid and antigen. Final complement titration to determine exact unit of complement used in test was done along with the test by adding 2 drops of complement from original master tubes to 1 drop of diluent in each well.

The plates were incubated at 4°C for 15-18 hours. Thereafter they were placed at 37°C for a few minutes before adding one drop (0.025 ml) of sensitized sheep cells per well. The plates were incubated at 37°C for 30 minutes and shaken at ten minute intervals. They were then placed at 4°C to allow cells to settle. A reading of 4 (no hemolysis) or 3 (25% hemolysis) was taken as positive. Reading of 2 (50% hemolysis) and less were negative.

Results obtained by the CF tests are shown in Tables 20 a, b, c. For simplicity only the titres of antibodies are given. The results are expressed as the reciprocals of highest dilutions of the MAP's fixing complement. A four-fold or greater difference between homologous titres was considered significant. There are three

TABLE 20a

RESULTS OF CROSS COMPLEMENT - FIXATION TEST OF ORUNGO VIRUS STRAINS: SUCROSE ACETONE ANTIGENS - SINGLE SHOT MAFs

ANTIGEN	IMMUNE MOUSE ASCITIC FLUIDS							
	11306	13019	30115	52302	60784	60818	60974	UgMP359
11306	32*	16	8	16	16	16	0	16
13019	8	32	8	4	4	32	0	32
30115	32	32	16	16	8	32	0	32
52302	16	4	8	32	4	32	0	32
60784	8	4	4	4	16	16	0	8
60818	64	64	16	16	8	32	0	32
60974	0	0	0	0	0	0	16	0
UgMP359	0	0	0	0	0	0	0	16

* Highest dilution of MAF giving at lease 3+ fixation.

TABLE 20b

RESULTS OF CROSS-COMPLEMENT-FIXATION TESTS OF ORUNGO VIRUS STRAINS-SUCROSE-ACETONE ANTIGEN - TWO-SHOT MAFs

ANTIGEN	IMMUNE MOUSE ASCITIC FLUID							
	11306	13019	30115	52302	60784	60818	60974	UgMP359
11306	64*	32	32	32	32	16	0	32
13019	64	64	16	32	32	32	0	128
30115	64	32	64	32	32	16	0	128
52302	64	16	16	32	64	4	0	64
60784	64	16	8	32	32	4	0	32
60818	32	128	64	32	128	64	0	256
60974	0	0	0	0	0	0	32	0
UgMP359	32	16	16	0	8	16	0	32

* Highest dilution giving at least 3+ fixation.

TABLE 20c

RESULTS OF CROSS COMPLEMENT-FIXATION TEST OF ORUNGO VIRUS STRAINS-SUCROSE ACETONE-ANTIGENS - FOUR-SHOT MAFs.

ANTIGENS	IMMUNE MOUSE ASCITIC FLUIDS							
	11306	13019	30115	52302	60784	60818	60974	UgMP359
11306	128*	64	128	32	128	16	0	64
13019	64	128	128	16	128	64	0	128
30115	64	128	128	32	128	32	0	128
52302	64	64	64	16	128	16	0	128
60784	64	32	32	8	128	16	0	64
60818	64	256	512	32	256	64	0	512
60974	0	0	0	0	0	0	64	0
UgMP359	64	64	32	8	64	32	0	32

* Highest dilution giving at least 3+ fixation.

significant observations from the results of cross-CF reactions. It is obvious from these results, that virus H60974 is not a strain of Orungo virus. In all reactions between the H60974 systems and the other virus strains, no cross-reactivity was demonstrated. This further confirms the results of the screening test. Secondly, with one-shot MAF (specific) the Uganda strain of Orungo virus showed a one-way cross reaction with the antigens of the other strains. Its antigen failed to react with the one-shot MAFS produced against the other strains. However, with broader reacting MAFS (hyperimmune) greater cross reactivity was noticed, until the 4-shot MAF level, when the Uganda strain showed complete 2-way cross-reactivity. Finally, strain H60818 consistently showed a broader reacting system than any of the other strains. Using the Uganda prototype strain as a basis for differentiation, it appears that strain 52302 is slightly different from the other strains. All the other strains are of human origin, while strain 52302 is an arthropod isolate.

The detail result of the CF tests are shown in Figs. 18 a, b, c.

4.9.3 Hemagglutinating property of Orungo virus as a method of antigenic analyses

Sucrose acetone extracted antigens of six strains of Orungo virus including the prototype were employed in HA tests against goat, goose, sheep, Patas and Rhesus monkey and human O erythrocytes.

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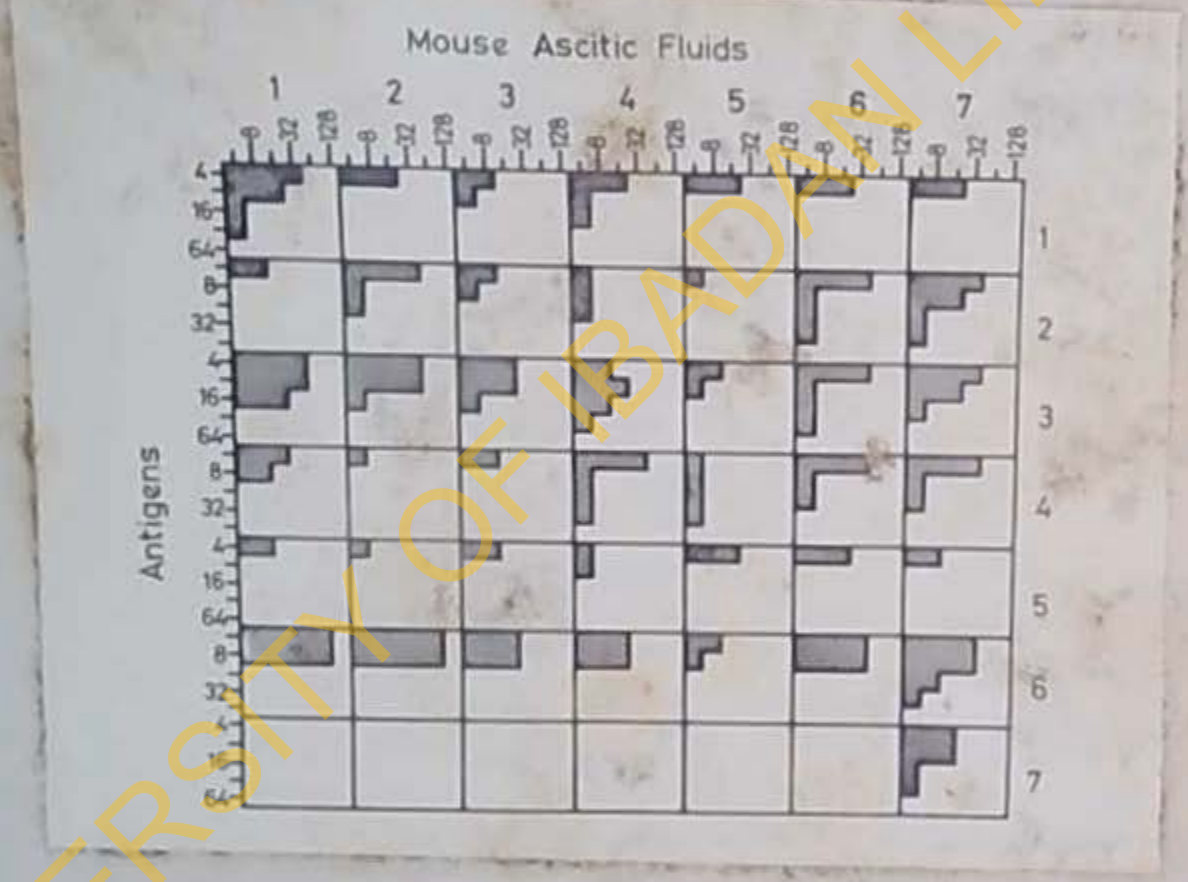
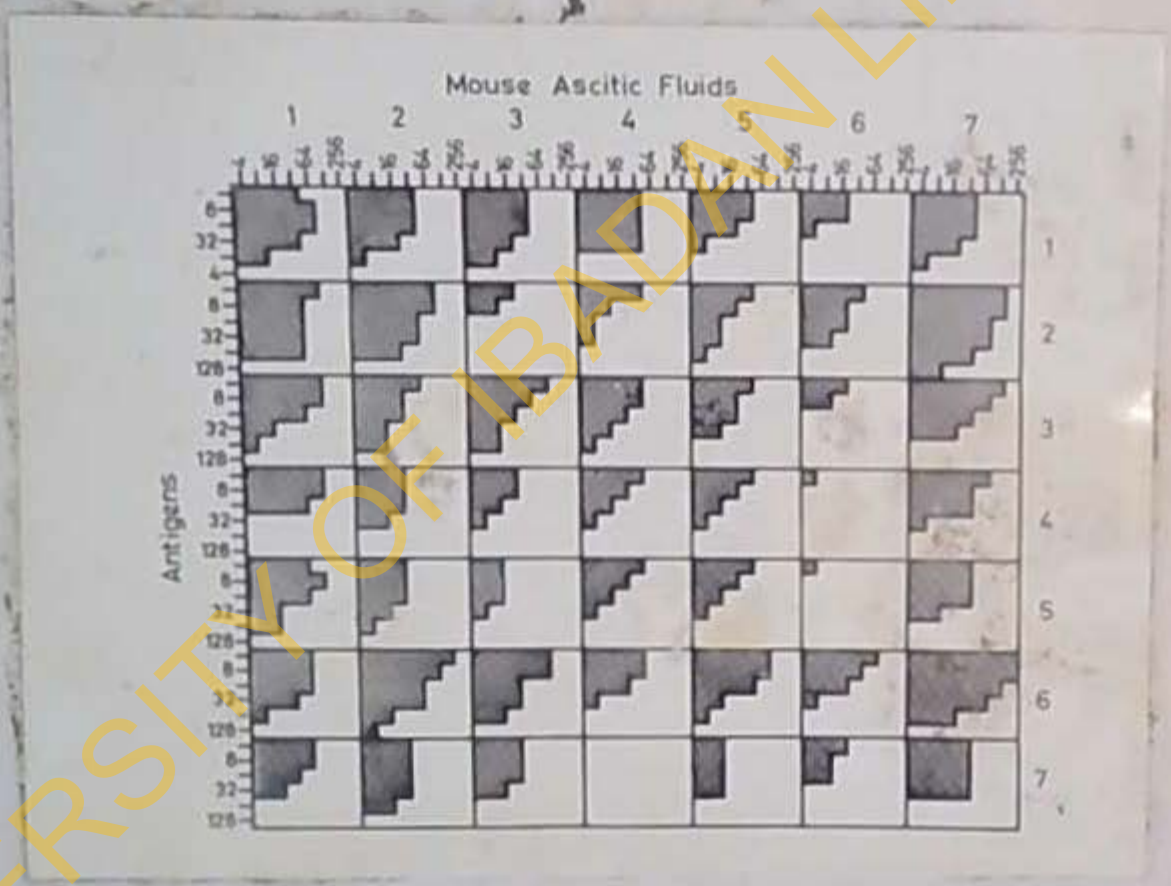
Fig. 18a



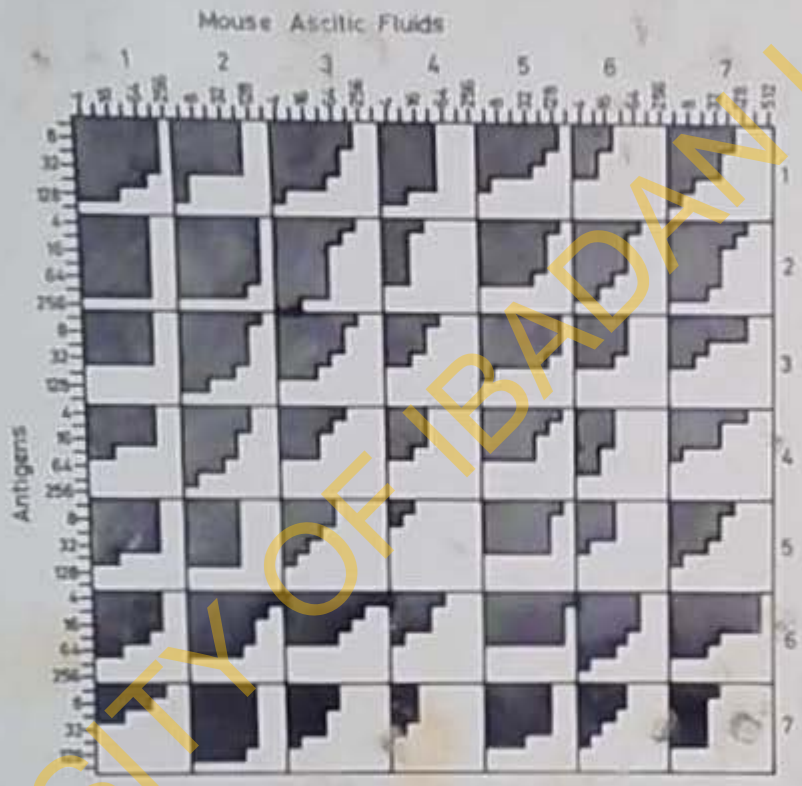
Immunological relationships among Orungo virus strains in complement fixation test. Sucrose acetone antigen versus 1 shot mouse ascitic fluids. (1 = H11306; 2 = H13019; 3 = H30115; 4 = AR52302; 5 = H60784; 6 = H60818; 7 = UgMP 359).

Fig. 18b



Immunological relationships among Orungo virus strains in complement fixation tests. Sucrose acetone antigen versus 2 shot mouse ascitic fluids. (1 = H11306; 2 = H13019; 3 = H30115; 4 = AR52302; 5 = H60784; 6 = H60818; 7 = UgMP 359).

Fig. 13c



Immunological relationships among Orungo virus strains in complement fixation tests. Sucrose acetone antigen versus 4 shot mouse ascitic fluids. (1 = H11306; 2 = H13019; 3 = H30115; 4 = AR52302; 5 = H60784; 6 = H60818; 7 = UgMP 359).

Tests were carried out according to the method of Clarke and Casals (op. cit.). Serial 2-fold dilutions of antigens in 0.4% bovine albumin (BA) were prepared. The antigen dilutions were tested at pH range of 5.8 to 7.2 at 4°C, room temperature (23°C ± 2°C) and 37°C. The erythrocytes were used in a final dilution of 1:240 of packed cells.

The HA titrations were performed in plastic plates by the microtiter method. The last well in each row contained equal volume of diluent and appropriate red cells as controls. Three sets of plates were incubated at the different temperatures to determine the suitable temperatures to incubate the test.

No hemagglutinating activity was detected when sucrose acetone extracted antigen for the six strains of Orungo virus (even after protamine treatment) were tested against goose, goat, sheep, Patas and Rhesus monkey, and human O erythrocytes. Similarly, hyperimmune MAFS prepared against the strains, (starting dilution 1:10) did not inhibit agglutination by hemagglutinnins (4-8 units) for representative viruses of the major virus groups.

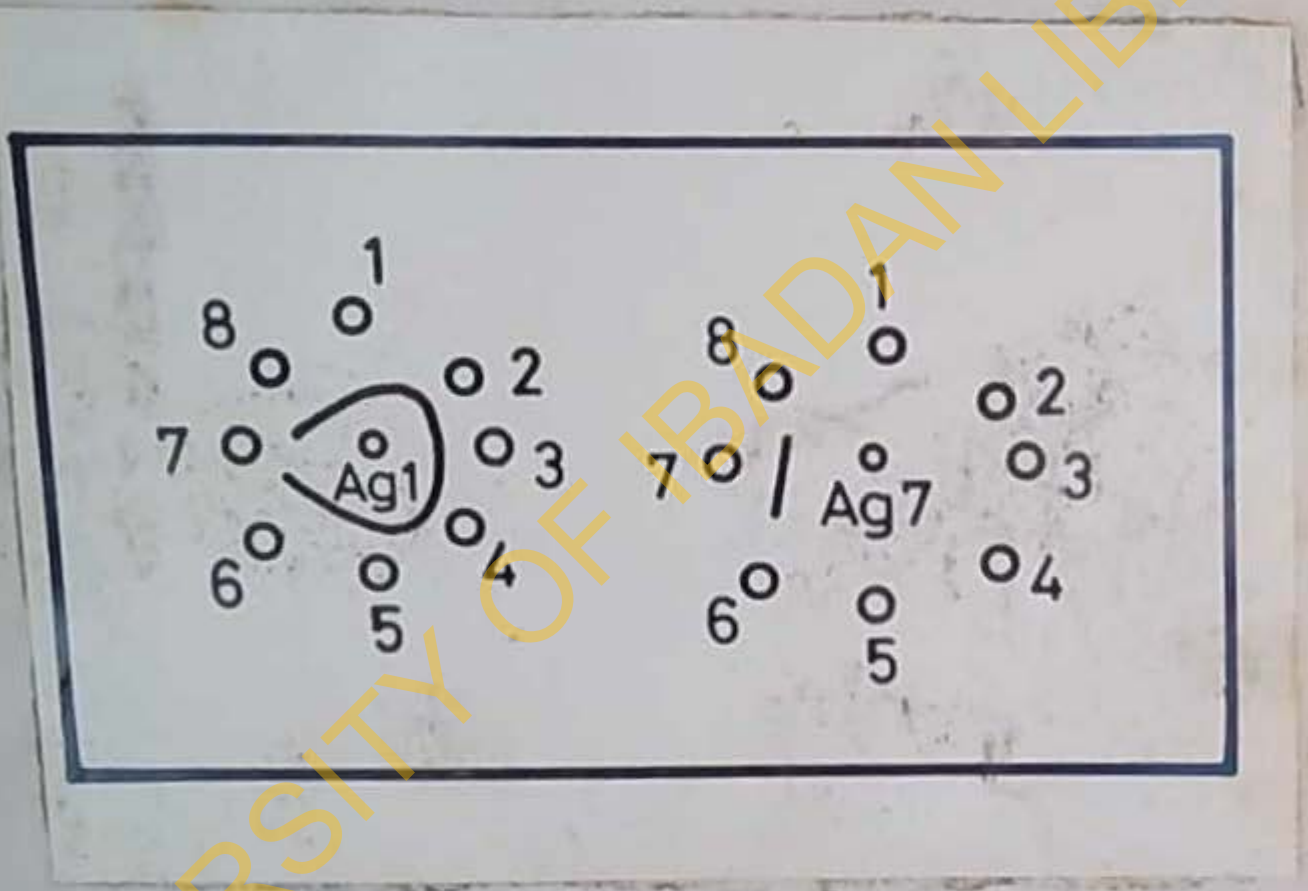
4.9.4 Agar-gel precipitation studies with Orungo virus strains

The agar-gel precipitation test of Ouchterlony was employed in the comparison studies of Orungo virus strains. Eight strains including H60974, found earlier on to be a strain of Tataguine virus

were used. Wells were cut into the layered agar with Gelman punch having eight peripheral and one central well cutters. The cut pieces of agar were removed from the wells by suction. Undiluted sucrose-acetone extracted, or 10% infected mouse brain saline suspended antigen were reacted with undiluted and uninactivated DMAFs. When central wells contained the antigens the peripheral wells were filled with the DMAFs and vice-versa. The slides were incubated at room temperature in humidified chambers and observed daily until precipitin lines were visible. Slides showing precipitin lines were photographed wet or stained for permanent record. Staining of slides was carried out as follows: slides were washed for several hours in normal saline with 3-4 changes of saline. The slides were next washed in distilled water for 30 minutes. Wet lintless papers were placed on the agar surface and left to dry overnight at room temperature. When the slides were dried, the lintless papers were removed and the slides stained with Thiazine Red dye for 5-10 minutes. Excess stain was removed with three successive washings in 5% acetic acid. The stained slides were again covered with lintless paper and left to dry at room temperature.

Figure 19 shows the schematic representation of precipitin lines obtained in the comparison studies with agar-gel precipitation test. Precipitin lines developed between crude antigen preparations and DMAFs but not with sucrose-acetone antigen. A single

Fig. 19



Representative results of agar-gel precipitation tests.

Central well contains antigen, outer wells, MAFS.

(1) = Orungo prototype strain UgMP 359, (2) = H11306,

(3) = H13019, (4) = H30115; (5) = AR52302; (6) = H60818,

(7) = H60794, (8) = H60818.

precipitin line of complete identity developed with 7 strains of Orungo virus. Precipitin lines developed to the strain H60974 only in homologous reactions.

Crude antigen preparations and IMAF of the strain H60974 were reacted with strain of Tataguine virus H9963 (antigens and MAF). Precipitin lines developed between strainsH60974 and the Tataguine virus system, but not with the other Orungo virus strains, Fig. 20. This confirms the results of CF tests that strain H60974 is Tataguine virus.

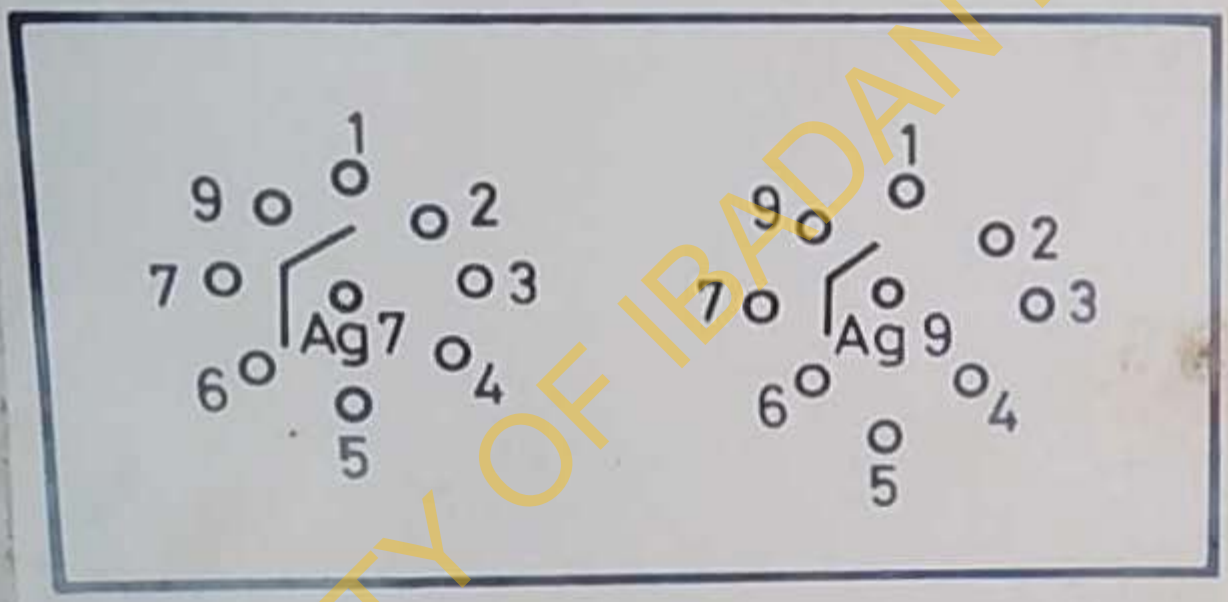
The microtitre modification of Ouchterlony test as described by Auernheimer and Atchley (op. cit.) was also used in the comparison test. This was reported to be more sensitive than the original Ouchterlony test. Similar results as obtained for the Ouchterlony test were recorded.

4.9.5 Neutralisation tests with Orungo virus strain

Two techniques of neutralization tests were employed with Orungo virus strains. These were the constant-serum-varying-virus dose, and the constant-virus-varying serum dose techniques. All neutralization tests were carried out in 2-3-day old mice.

In the constant-serum-varying-virus dose technique, equal volume of MAF and 10-fold serial dilutions of each virus were incubated together for 60 minutes at 37°C. After incubation, the virus-MAF mixture were placed in ice bath for inoculation into

Fig. 20



Representative result of agar-gel precipitation tests. Central wells contain antigens, outer wells, MAPS. (1) Orungo prototype, strain UgMT 359, (2) H 11306, (3) H 13019, (4) H 30115, (5) AR 52302, (6) H 60818, (7) H 60974, (9) Nigerian topotype of Tataguine virus, strain H 9963.

2-3-day old baby mice. 0.02 ml of each mixture was inoculated IC into baby mice which were observed for 14 days. Titration end points from the neutralization indices were calculated and determined by the method of Reed and Muench (op. cit.).

For the constant-virus-varying serum technique, equal volume of a known virus dose (100 LD_{50}) and 2-fold serial dilution of MAF were incubated for 60 minutes at 37°C. Other procedures were as described for the constant-serum-varying virus dose technique. Cross-neutralization tests were performed by reacting each virus strain with the homologous and heterologous IMAF. Table 21 shows the neutralization indices from results of cross-neutralization tests by the constant-serum-varying-virus dilution technique. Differences between strains were considered significant if heterologous and homologous titres differed by at least 1.0 dex. Using the prototype, UgMP 359 strain as a basis for reference, only strain H 13019 showed a two-way cross N reactivity with the prototype. The MAF to the prototype neutralized 1.4 dex and 1.3 dex of the homologous and heterologous virus strains respectively. Similarly, MAF 13019 showed neutralising indices 1.3 and 1.9 dex to homologous and heterologous viruses respectively. The MAF to the prototype neutralized 1.7 dex and 2.9 dex of strain H 60784 and H 60818 viruses respectively, however MAF 60818 neutralized only 0.7 dex of the prototype while there was no cross-protection between the MAF 60784 and the prototype virus strains. The arthropod isolate

TABLE 21

NEUTRALISATION INDICES OF CROSS-NEUTRALISATION TESTS WITH ORUNGO VIRUS STRAINS

Virus Strains	NEUTRALISATION INDICES (IN DEX) Immune House Ascitic Fluids (IHAFs).						
	11306	13019	52302	60784	60818	60974	UgMP 359
11306	2.0	0	0	0	0	0	0
13019	0	1.3	0	0	1.3	0	1.3
52302	0	0.6	2.8	2.3	0.9	0	0
60784	1.8	2.8	2.8	2.8	1.7	0	1.7
60818	0.4	3.9	0	1.0	2.5	0	2.9
60974	0	0	0	0	0	4.0	0
UgMP 359	0	1.9	0	0	0.7	0	1.4

from Nigeria was more closely related to strain H 60784, as both virus systems cross-protected almost titre for titre. Strain H 11306 showed only a one-way cross-reaction with strain H 60784, with its MAF neutralizing 1.8 dex. Strain H 60974 was included to confirm results obtained by the CF and AGD tests.

Using the constant-virus-varying serum technique, a four fold difference between homologues and heterologous serum dilutions neutralizing 100 LD_{50} of virus, was considered significant in differentiating virus strains. Greater cross-reactivity was observed between the virus strains according to the results obtained by this method (Table 22). Strain H 11306, which in the former method show only a one-way cross-reaction with strain H 60784, now showed a two-day reaction, although a significant difference was noticed in the homologous and heterologous reactions with UgMP 359 the prototype strain. Similarly strain AR 52302 whose MAF cross protected strain H 60784 by the constant-serum varying virus technique, now showed cross-reactivity with all the other strains, although, reaction with strain H 60784 was greatest.

4.10 Serological surves for Orungo virus neutralizing antibodies in Nigeria

A survey for neutralizing antibodies to Orungo virus was conducted on sera from man and animals in 17 locations of 4 ecological zones. Figures 22 shows these locations. Venous blood was

TABLE 22

RECIPROCAL OF SERUM DILUTIONS NEUTRALISING 2 DEX OF ORUNGO VIRUS

Virus Strains	Immune Mouse Ascitic Fluids (IMAFs)						
	11306	13019	52302	60784	60818	60974	UgIP 359
11306	25	16	12	15	25	0	7
13019	8	40	7	8	50	0	17
52302	18	5	20	18	25	0	6
60784	22	50	20	60	35	0	15
60818	18	17	4	25	50	0	4
60974	0	0	0	0	0	64	0
UgIP 359	5	35	10	10	12	0	20

Fig. 21

Map of Nigeria showing sites of collection of human and animal sera.

collected from humans into sterile syringes or vacuum tubes. Sera were separated and stored in plastic tubes in liquid nitrogen or kept cold in wet ice. On return to the Ibadan Laboratory, sera were transferred to a mechanical freezer (-20°C). The number, source and date of collection of human sera tested are shown in Table 23. Details of methods of collection and processing of monkey sera have been described by Monath et al. (1974), and of domestic animal and other wild animal sera by Kemp et al. (1971). Birds were collected in mist nets in Ibadan and brought back to the Laboratory, where they were bled by cardiac puncture. Sera were separated by centrifugation and stored at -20°C until tested. The number location and date of collection of animal sera tested are shown in Table 25. The Ib H 13019 strain of Orungo virus was used in this survey at the 7th mouse brain passage level.

For the surveys, neutralization (N) tests were performed using a constant serum-constant-virus dose technique in 2-4 day old Swiss white mice. Undiluted sera were inactivated at 60°C for 30 minutes. An equal volume (0.1 ml) of virus suspension containing an estimated 100 LD_{50} was added to each serum and the mixture incubated at 37°C for one hour. Litters of 6 baby mice were inoculated IC each mouse receiving 0.02 ml of individual virus-serum mixture. Inoculated mice were observed for 14 days. The actual challenge dose ranged from 40 LD_{50} to 180 LD_{50}. A positive

TABLE 23

SOURCE, DATE OF COLLECTION AND NUMBER OF HUMAN SERA TESTED FOR ORUNGO VIRUS NEUTRALIZING ANTIBODIES IN NIGERIA

VEGETATION ZONE	LOCALITY	DATE OF COLLECTION	NUMBER OF SERA
Rain Forest	Abeokuta	1973	159
	Ibadan	1973-1974	157
	Onitsha	1973	111
	Nnewi	1971	32
	Afikpo	1974	41
	Uyo	1974	141
Derived Savannah	Enugu	1974	33
	Okaoga	1971	94
Southern Guinea Savannah	Shaki	1973	19
	Nupeko	1971-1972	42
	Lafia	1973-1974	38
Northern Guinea Savannah	Jos	1970-1972	39
	Shendam Area*	1973-1974	293
Total			1197

*Shendam Area: Shendam, Mabudi, Sabongida, Mlokhan, and Makat.

TABLE 24

RESULTS OF NEUTRALIZATION TESTS FOR ONUNGO VIRUS ANTIBODIES, URMAN SERA, NIGERIA AGE (YEARS)

Vegetation Zone	0-9	10-19	20-39	40+	Total	Standardized rate for age
Rain Forest	2/145*(1.3)**	19/124(15.3)	59/301(19.6)	20/71(28.2)	100/641 (15.6)	17.0%
Derived Savannah	2/17 (13.3)	3/26 (11.5)	17/71 (23.9)	7/13 (53.8)	29/127 (22.8)	21.3%
Southern Guinea Savannah	0/5 (0)	4/37 (10.8)	17/36 (47.2)	7/21 (33.3)	28/99 (28.2)	29.9%
Northern Guinea Savannah	8/31 (25.8)	14/55 (25.4)	59/163 (36.2)	39/81 (48)	120/330 (36.4)	34.3%
Total	12/198 (6.1)	40/242 (16.5)	152/571(26.6)	73/186(39.2)	277/1197(23.1)	
Standardized rate for geographic zone	9.2%	17.3%	26.9%	36.0%		

* number positive/total number tested.

** percentage positive.

result was recorded if 6 of 6, 5 or 6 or 5 of 5 mice survived at the close of the experiment. All other results were recorded as negative.

Of a total of 1197 human sera tested, 277 (23.1%) were positive for Orungo virus N antibody (Table 24). The standardized prevalence rates for age and vegetation zones were not significantly different from the non-standardized rates, however the standardized rates were used in the analyses. The highest prevalence rate (34.2%) was found in the northern guinea savannah zone. The prevalence of antibody in the derived savannah zone (24.3%) was similar ($p > 0.25$) to that in the southern guinea savannah zone (29.9%) but significantly lower ($P < 0.0001$) than the prevalence in the northern guinea savannah zone. No significant difference ($0.25 > p > 0.1$) was found in the prevalence rates between the southern and northern guinea savannah zones. The lowest prevalence rate (17.0%) of Orungo virus (N) antibody was found in the rain forest, and was significantly lower than that in any other zone. Apart from the rain forest zone, there were no significant differences in prevalence rates between adjacent zones, but an increasing trend in prevalence rate was observed from the wet forested areas to the drier savannah regions.

In all areas, the prevalence of antibody significantly increased with age, this indicated endemic presence of the virus. The lowest rate (9.2%) was found in the 0-9-year age group. This

TABLE 25

RESULTS OF NEUTRALISATION TESTS FOR ORUNGO VIRUS ANTIBODIES IN ANIMAL SERA IN NIGERIA

Species	Place of collection	Date of collection	Number positive over number tested
Domestic animals:			
Cows	Ibadan, Jos, Kano, Maiduguri	1967-1969	14/99
Goats	Ibadan and Maiduguri	1970	0/48
Horses	Zaria and Lagos	1970, 1973	0/12
Sheep	Ibadan	1970-1971	23/44
Camels	Maiduguri	1970	0/10
Total			37/213
Wild animals:			
Rodents:			
Arvicanthus niloticus	Ibadan, Bassa (Jos), Kwaro	1969-73	0/12
Cricetomys gambianus	Ibadan, Nupeko	1970-71	0/15
Lophuromys sikapusi	Ibadan	1969	0/1
Mastomys natalensis	Ibadan, Shondan	1970, 1973	0/18
Rattus rattus	Ibadan	1967-68	0/12
Uranomys ruddi	Ibadan	1969	0/12
Primates:			
Galago demidovii	Dada	1969	0/20
Cercopithecus mona	Nupeko	1971	6/24
C. aethiops tantalus	Nupeko	1971	2/8
C. nictitans martini	Nupeko	1971	1/6
Chiroptera:			
Eidolon helvum	Ibadan	1970	0/5
Epomops franqueti	Nupeko	1971	0/3
Insectivores:			
Atelerix albiventricus	Dada	1969	0/66
Crocidura spp.	Ibadan	1969-71	0/8
Birds:			
Ploceus nigerrimus	Ibadan	1971	0/15
Ploceus cucullatus	Ibadan	1971	0/5
Pycnonotus barbatus	Ibadan	1971	0/2
Turdus pelios	Ibadan	1971	0/3
Streptopelia senegalensis	Ibadan	1971	0/5
Total wild animals			2/273
Total domestic and wild animals			46/486

was significantly lower ($p < 0.001$) than the rate of 17.3% in the 10-19 year group. In the 20-39 year group, 26.9% had N antibodies to Orungo virus, while 36.8% of the above 40-year age group were positive for N antibodies to Orungo virus.

Forty-six or 9.6% of the 486 animals sera tested were positive for N antibodies to Orungo virus (Table 25). Of the 213 domestic animal sera tested, 14 from 99 cows and 23 from 14 sheep were positive. All other sera from goats, horses, and camels were negative. While only sheep from Ibadan area were sampled, 11 of 37, 1 of 11, and 2 of 20 cow sera collected in Ibadan, Kano and Maiduguri respectively were positive for antibodies to the virus. None of the eleven cow sera from Jos was positive. Nine, i.e. 26% of 38 monkey sera from Mupeko tested for Orungo virus N antibody were positive. All other wild animal and bird sera were negative.

4.11 Clinical symptoms of Orungo virus infection

To date, nine strain of Orungo virus have been isolated in Nigeria. One of these was from a pool of mosquitoes Aedes dentatus spp., the rest are human isolates. Although most of the Orungo virus isolates were of human origin, little information is available about the clinical symptoms of Orungo virus infection. This is because all the human isolates were obtained from children reporting sick at the Out Patient Clinics where little or no information on clinical symptoms of febrile illness of short

duration is obtained. Invariably, most of the patients never report back, as they recover from such illness in spite of rather than as a result of the treatment they received. Most of these illness are diagnosed as malaria or at best pyrexia of unknown origin. The generally described symptoms in association with the cases yielding Orungo virus isolates were fever (38°C-40°C) of 3-7 days duration and generalised body pains. However, a few cases got admitted because of other complications. One of these was reported by Familusi et al (op. cit.). A 2-year old Nigerian girl presented with persistent fever and diarrhea of one month's duration, despite treatment with antimalarials and antibiotics. On admission, temperature was 40°C, with weakness of the lower extremities. The diarrhea subsided with treatment, but the fever persisted for five days. Orungo virus was isolated from the blood sample collected on admission. The weakness of the lower extremities improved on recovery. However the patient was discharged before a convalescent serum sample could be taken. Fabiyi et al. (op. cit.) also described three outbreaks of an epidemic of human illness in Jos, Plateau State of Nigeria in 1972. The illness was characterised by nausea, myalgia, headache and fever of 3-7 days duration. Although no virus was isolated from samples collected during the outbreak, over 60% reacted with very high CF antibody titres (1:32-1:128) to Orungo virus only. Two deaths were reported in individuals with the described symptoms

at the time of the epidemic. The relationship of these deaths to the reported outbreaks in unclear. Monath et al. (op. cit.) reported the isolation of strains of Orungo virus from the blood of patients collected during another epidemic of human illness in the Anambra State of Nigeria. The symptoms of the reported epidemic were fever, headache, and myalgia. Several reports of suspected outbreaks of yellow fever infection were investigated in 1973 and 1974 at Mabudi in the Plateau State of Nigeria. Results of the investigation showed that Orungo virus was also active at or about the same time as the yellow fever outbreak (Tomori et al, op. cit.). Two clearly defined clinical symptoms were recorded during these outbreak; classical yellow fever symptoms-biphasic febrile response with jaundice, hematemesis and melena; and the usual fever of short duration, myalgia and headache as reported for the two previous outbreaks of Orungo virus. Fagbami et al. (op. cit.) isolated Orungo virus from a 14-year old child at Abeokuta in Ogun State of Nigeria, during an outbreak of a dengue-like illness involving adults and children. Other viruses isolated during the outbreak were chikungunya and dengue. The symptoms of this outbreak included fever, headache, muscle, joint and retro-orbital pain, anorexia, rash, lymphadenopathy and leucopenia. In a case report (Ogunlesi, unpublished data), a 50-year old male developed a sudden illness with fever, headache,

pain and weakness in his limbs. Conjuctivitis was noticed two days prior to the onset of fever. The pain in the limb subsided within 24 hours but fever, headache and weakness in the limbs continued for seven days. Daily temperatures were between 39°C and 40°C. Other significant symptom during the illness was skin tenderness especially around the trunk. A fine papular rash appeared on the face, chest and abdomen on the third day of the illness and this lasted for two to three days. There was no pain or swelling of the joints nor was there any abnormality found in the chest, abdomen and nervous system. Acute and convalescent serum samples collected 4 days and 13 days respectively from onset of illness were checked for virus isolation and serology. No virus was isolated from the two samples, however acute sample was positive for Orungo virus CF antibody to a titre of 1:8 and convalescent sample to a titre of 1:256. The samples were also tested by CF against thirty other viruses and found negative. In neutralization test against Orungo virus, the acute and convalescent samples neutralized 0.8 dex and 3.7 dex respectively of Orungo virus. These results would indicate that Orungo virus was most probably responsible for the described illness.

It would appear that fever, headache and myalgia or generalized body pain are the common symptoms of Orungo virus. In two cases, Familusi et al. (op. cit.) and Ogunlesi (op. cit.), where detailed

clinical symptoms were recorded, weakness of the limbs was described. On both occasion the weakness of the extremities improved on recovery. Conjuctivitis was recorded only on one occasion. Orungo virus can therefore be described as a febrile disease of short duration, characterised by headache, myalgia and possibly weakness of the limbs. In this respect it resembles other viral diseases in Nigeria and a proper recording of the most detail clinical symptoms coupled with virus isolation and antibody studies are needed to differentiate it from these other virus diseases., as well as the ubiquitous malaria.

4.12 Identification of virus strain H 60974 as Tataguine virus

Following the differentiation of strain H 60974 from the other Orungo virus strains, and the positive CF reaction with Tataguine MAF in the screening tests, it was reacted in cross CF tests with sucrose acetone antigen and MAFS of two known Tataguine virus strains, H9963 and H39482. Cross neutralisation tests with Tataguine and Orungo MAFS were also carried out. Positive and negative controls were included in the tests.

The results of the cross-CF and cross-(N) tests are presented in Tables 26 and 27. Strain H 60974 showed complete antigenic identity with the two Tataguine virus strain.

TABLE 26

RESULTS OF CROSS COMPLEMENT FIXATION TESTS BETWEEN VIRUS H60974 AND 2 STRAINS OF TATAGUINE AND ORUNGO VIRUSES.

ANTIGENS	MAPS				
	H9963 (TATAGUINE)	H39482 (TATAGUINE)	H60974	H13019 (ORUNGO)	UgMP 359 (ORUNGO)
H9963	64/16	64/16	64/8	0	0
H39482	32/8	64/32	32/16	0	0
H60974	32/32	64/16	64/64	0	0
H13019	0	0	0	128/64	32/8
UgMP 359	0	0	0	128/16	32/32

RESULTS OF CROSS-NEUTRALISATION TESTS BETWEEN H60974, TATAGUINE AND ORUNGO VIRUSES.

VIRUS	Titre in Dex/0.02	ASCITIC FLUID LNT		
		H60974	H9963 (Tataguine)	H13019 (Orungo)
H60974	6.0	4.0	3.2	0
H9963	5.6	2.5	2.8	0
H13019	5.5	0	0	1.8

CHAPTER 5

DISCUSSION

Certain characteristics of Orungo virus were investigated by biochemical and biophysical sero-immunologic techniques.

By electron microscopy, Orungo virus was found to develop in the cytoplasm of infected cells as an unenveloped particle with an electron-dense core. In association with its development and maturation, there were specific viral granular matrices and filaments. This particular type of morphology and morphogenesis, has only been described for the orbivirus taxonomic group, Murphy et al. (op. cit.); into which Borden et al. (op. cit.) have placed Orungo virus. These filaments may represent viral subunits arranged in anomalous manner. The virus particle diameter of 63 nm is within the 62 - 80 nm range reported for other orbiviruses, Murphy et al. (op. cit.). Although none of the methods resulted in optimal resolution of particle capsid structure, the presence of a hexagonal electron transparent zone basal to the capsomeres suggests the typical icosahedral construction (T = 3) of the major capsid layer. Orungo virus is exceptional amongst the other orbiviruses in the resistance of its outer coat layer. With other orbiviruses, there is usually difficulty in maintaining the integrity of the indistinct outer coat which obscures the major capsid layer. For example with blue tongue virus, the outer coat is lost as virus

moves through an equilibrium centrifugation gradient, so that banded virus has the precise T = 3 capsid construction. Orungo virus particles never were found in a state where their outer coat layer was removed and capsid layer intact. Trypsin treatment, for example, reduced particles to their inner layers (core particles). Questions concerning the stability of Orungo virus relative to other orbiviruses and how stability may be related to the nature of the outer coat layer of the virus particles, remain to be answered. The exceptional localization of Orungo virus particles around intact mitochondria must reflect differences in the cytoplasmic environment in different parts of the infected cell. No explanation is available, but further study of this phenomenon is warranted.

Thermal inactivation of Orungo virus at 56°C, 37°C and room temperature (22°C ± 2°C) was of the two component curve, each curve following a first order kinetics. This phenomenon appears to be a general characteristics of animal viruses Joose (1953). At the three levels of inactivation, there is a rapid denaturation, of Orungo virus, more than 50% of virus infectivity being lost within 10 minutes at 56°C and within the first 24 hours at 37°C and room temperature even when virus was suspended in medium containing 5% serum. This could explain the inability to isolate Orungo virus from field samples kept at room temperature for over 24 hours, as was usually the case in most of the suspected Orungo virus disease

outbreaks. However at 4°C storage virus infectitity is retained for a considerable length of time. Thus keeping samples just at 4°C could result in a higher number of Orungo virus isolates from field samples.

The CF antigen of Orungo virus is labile at 56°C, but stable at 37°C and room temperature. Infectivity of Orungo virus is not significantly affected by lyophilization and subsequent storage at 4°C for 18 months.

The inactivation of Orungo virus by UV irradiation affects the infectivity but not the CF antigen. Orungo virus is only slightly sensitive to sodium desoxycholate, ether and chloroform, and was more resistant to these reagents than yellow fever virus (a togavirus). Resistance to sodium desoxycholate, ether and chloroform has often been correlated with an unenveloped virus particle. Electron microscopic studies of Orungo virus confirms the naked virion nature, and absence of a membrane in this virus. In similar studies, Borden et. al. (op. cit.) suggested that the slight but reproducible solvent sensitivity of the orbiviruses may represent a fundamental virion property or laboratory artefact. At low mouse brain passage, Orungo virus sensitivity to the lipid solvents was 0.8 dex - 1.0 dex; with increasing passages, Orungo virus became more sensitive to these agents. Similar results were reported for Colorado tick fever virus by Borden et. al. (op. cit.)

This solvent sensitivity may be related to host cell contaminant or to lipid in the virus itself. Verwoed, (1969) reported that purified blue tongue virus, an orbivirus, contained 2% lipid. Further investigation is necessary to determine the source of and the quantity of lipids in Orungo virus.

Orungo virus is sensitive to treatment with chemical reagents such as BPL and formalin. Under the same experimental conditions a lower concentration of formalin inactivated Orungo virus than was required by BPL. However, using formalin and BPL at 37°C, LoGrippo et. al. (1954) reported that BPL inactivated rabies, eastern equine, and M-M strain of murine encephalomyelitis viruses more readily and at lower concentration than are required by formalin. Whether Orungo virus being an orbivirus, differs from these other viruses or the temperature of inactivation affects the property of the reagents remains to be determined.

Orungo virus is labile at pH 3.0, but stable between pH 5.0 and 7.0. Acid lability has been used as an aid in virus classification (Hamparian et. al. 1963). This is not a major taxonomic character, but it has been useful in separating human rhinovirus from enteroviruses, a separation subsequently confirmed by more fundamental physical differences (Tyrell, 1968). Orungo virus is strikingly labile at pH 3.0, no infectivity was found in undiluted specimens after treatment. Similar results were reported by

Borden et. al. (op. cit.) for the orbiviruses such as Colorado tick fiver, Irituia, bluetongue, Tribec, Tad Medani and Lebombo. In contrast reoviruses are stable over a wide range of pH values (Stanley, 1967).

The stability of Orungo virus to 5-iododeoxyaridine suggests that it may be an RNA virus. Similarly many of other viruses (blue tongue, Colorado tick fever and African horse sickness), of the orbivirus taxon possess a double-stranded RNA genome (Verwoerd et al. 1969; Green, 1970; and Oellerman et. al., 1970). Further biochemical tests to determine the nature of Orungo virus genome is warranted.

Following successive intracerebral passages in mice, Orungo virus became adapted and stabilized for the host from the sixth passage. The decrease in Orungo virus infectivity with increasing age at inoculation time of mice is a general observation with animal viruses, (Burgher, 1971; and Saikku and Brummer-Korvenkontio, 1973). The reason for the differences in age susceptibility of laboratory animals to virus infection is not definitely known, it is thought that certain receptors required for virus attachment are present to a higher level in the target organs of the young than in those of the adult. The poor growth in 10-day old animals which become sick, but recovered from an infection with Orungo virus cannot be solely attributed to poor feeding as animals continued to

feed, through the period of illness. Grossly, organs of recovered animals are smaller than those of the control uninfected. This aspect of Orungo virus infection in mice needs further investigation with particular reference to the effect of infection in pregnancy. It will then be possible to determine if there is placental transfer of the virus to the fetus and the effect of such on the developing fetus.

The organ distribution of Orungo virus in Swiss albino mice shows that the brain is the target organ. Although viremia and viruria were demonstrated, the level of virus in the body fluids as well as organs such as the heart, lung, liver, kidney and spleen were in general significantly lower than in the brain. This is more clearly brought out in infected 10-day old mouse, where virus multiplication even in the brain was minimal. At the lower infecting dose, higher viremia and viruria level were obtained, possibly as a result of the increased AST.

The spread of Orungo virus to the different sections of the brain following inoculation into the left cerebral half is dependent on the virus dose. Using 10 LD_{50}, virus multiplication and cellular infection is restricted to the cerebral halves, with the cerebellum showing low virus infectivity at the terminal stages. With 1000 LD_{50} of virus as infecting dose, virus was detected in all brain sections. The restriction of pathology in infected mice to the brain and the absence of extra-neural Orungo virus specific

immunofluorescence further confirms the neurotropic nature of Orungo virus infection in mice. Further evidence for the neurotropic nature of Orungo virus infection is shown by the inability to infect mice by the SC and IP routes. In addition, only mice infected by the IC route developed neutralizing antibodies to Orungo virus.

Hamsters are susceptible to Orungo virus infection by the IC, SC and IP routes. However, viremia was demonstrated only by the IC and IP routes following infection of 2-day old hamsters. Evidence of infection in 3-week old hamsters inoculated by the IP and SC routes was the development of specific Orungo virus N antibodies.

Rabbits and lambs, do not circulate Orungo virus following infection with the virus by the IV, SC, IP or conjuctuval route of inoculation. They however develop specific Orungo virus N antibody: the rabbits following IV, IP and conjuctival routes, and the lambs following IV route of infection.

Two of the avian species, sparrows and baby chicks neither circulated Orungo virus nor develop antibodies to the virus following inoculation by the IV, SC or oral routes.

According to the World Health Organisation (1967) arbovirus are viruses which are maintained in nature principally or to an important extent through biological transmission between susceptible vertebrate hosts by hematophagous arthropods. They multiply and

produce viremia in the vertebrates, multiply in the tissue of arthropods and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation. Orungo virus has been isolated on three occasions from arthropods, and from the blood of vertebrates during a febrile infection. Low level viremia was demonstrated in laboratory mice and hamsters. However it was not known if arthropods acted as mechanical or biological transmitting agents. The demonstration of a possible transmission of the virus was hampered by the absence of a suitable host; mice are not infected by a SC inoculation of Orungo virus, and hamsters only develop antibodies following inoculation by the same route. Orungo virus was inoculated parenterally into mosquitoes, and following an extrinsic incubation period of 6-11 days, transmission was achieved by allowing these mosquitoes to "bite" on blood droplets. Moreover, virus titres in these mosquitoes ranged from 2.3 dex to 3.0 dex. None of the mosquitoes fed by the oral route on a virus-blood mixture transmitted the virus. Certain factors might have been responsible for this negative result. The low level (about 4.0 dex) of Orungo virus in the blood meal could account for failure of virus to establish in the mosquito. Schaeffer and Arnold (1954) suggested a viremia level of 4.5 dex and above as a high mosquito infecting potential. Even when the virus level is high, the ingested virus must survive anatomic, physiological and biochemical barriers in several organs of the

mosquito gut before progeny virus is delivered to the salivary secretions for possible transmission (Murphy, 1975). These barriers refered to as "gut barrier" or "threshold" must be overcome before virus replication takes place in the mosquito. Whether virus is actively destroyed by means unknown or die off gradually in the absence of favourable condition is not known, (Chamberlain and Sudia, 1961).

The length of incubation required to attain maximal transmitting efficiency is also important. The 11 days extrinsic incubation period was probably not long enough to attain enough virus for transmission by mosquitoes fed orally, as compared to mosquitoes inoculated intrathoracically in the vicinity of the salivary gland and which would therefore require a shorter incubation period.

The failure of mosquitoes infected either by thoracic inoculation or oral feeding to transmit Orungo virus to baby mice further lends support to previous observation that mice do not become infected by the SC route.

With the transmission of Orungo virus by Aedes spp. mosquitoes, the virus has satisfied the major criteria for classification as an arbovirus. However, a suitable animal host that will circulate virus is required to fully understand the natural transmission of the virus.

By treating infected Vero cells cultures with trypsin and regrowing the trypsinized cells, there was an increased sensitivity of vero cell cultures to infection by Orungo virus. Itoh et. al. (1970) have also reported increased sensitivity of vero cells for three strains of influenza and three of paramyxoviruses following treatment of the Vero cells with trypsin. The cultures were maintained with a medium modified by the addition of trypsin. Infectivity titres of the influenza and paramyxoviruses were higher in cultures with added trypsin than in normal Vero cultures. Although Itoh et. al. (op. cit.) reported no significant differences in the sensitivity of Vero cells to many other viruses, after trypsin treatment, it appears that the modification used increased the sensitivity of Vero cells to infection by Orungo virus. Since the biological property of Orungo virus passaged in trypsin treated culture was not different from mouse brain passaged virus, it is safe to suggest that the trypsin did not induce the emergence of new variants, but rather acted to increase the sensitivity of the cell culture to Orungo virus.

The release of Orungo virus from the monolayer culture into the fluid medium does not occur until complete CPE is achieved. Similar observations were reported for the other orbiviruses (Murphy et. al., 1971).

Orungo virus was adapted to BHK-21 cell culture without the need for sensitization of the cell culture. The phenomenum of late viral release into the fluid medium was also observed in BHK-21 cell cultures. By immunofloure scent studies virus particles were found located in the cytoplasm in focal masses initially and large punctuate masses at the late stage of infection.

The failure of Orungo virus to replicate in Aedes albopictus cell line could be due to non-adaptability of the virus to this particular cell line. Libikova and Buckley (1971), reported that a persistent infection in A. albopictus cells by Kemerovo virus was achieved over some 50 transfers of the carrier culture with only about 1% of cells being infected. Kemerovo virus is a tick-borne virus belonging to the orbivirus taxon (Borden et. al. op. cit.). Further transfers of Orungo virus infected A. albopictus cells may also reveal persistent infection of the cell line by Orungo virus or adaptability of the virus to this particular cell line.

Sucrose acetone antigens prepared from Orungo virus-infected mouse brain tissues failed to agglutinate goose, sheep, goat, day old chick, monkey and human O erythrocytes. Similar results using only goose erythrocytes with other orbiviruses have been reported by Borden et. al. (op. cit.). However, Sarmanova et. al. (1965) detected hemagglutinating activity in Kemerovo virus antigen

prepared in chick embryo and swine kidney cultures. Orungo virus cell culture antigen may well yield hemagglutinating activity; this needs further investigations.

One of the 10 strains of viruses isolated in Nigeria previously reported as Orungo virus by Monath et. al. (op. cit.), was found by CF test to be a strain of Tataguine virus. The other nine strains did not cross-react with over 150 different viruses belonging to the major arbovirus groups. In cross-CF tests, between seven Nigerian strains and the prototype Uganda strain, no significant differences were found in the reactions of the different strain. However it is of note that the Uganda strain antigen did not react with the specific (1 shot) MAFs prepared against the Nigerian strains. In addition, cross-reaction was demonstrated between the Uganda strain antigen and strain Ib AR52302 MAF only with the hyperimmune (4 shot) MAF of Ib AR52302 strain. Other strains cross reacted with the prototype from the 2-shot MAF stage onwards. It would therefore appear that strain Ib AR52302 is slightly different from the other strains.

By agar-gel diffusion test, a single line of identity was demonstrated between all the strains of Orungo virus, using crude antigen and hyperimmuned MAFs. However, with sucrose acetone antigens, no precipitin lines developed. Similar results were obtained with the more sensitive modification of Auernheimer and

Atchley (op. cit.). This would tend to suggest complete identity between all Orungo virus strains, or the presence of a common antigen. However the AGD test is known to be much less sensitive than the CF or neutralization test. Therefore, minute differences between strains will not be detected by the less sensitive test. It appears that sucrose-acetone extraction of Orungo virus antigens renders them unsuitable for precipitin tests. This is unusual, as with Bwamba-Pongola virus group (Tomori and Fabiyi, 1976) both crude and sucrose acetone antigen gave precipitin lines.

In neutralization tests greater differences were demonstrated than previously noticed in the CF tests. By the constant serum, varying virus neutralisation technique, Orungo virus strain H13019 was found to be more closely related to the prototype strain than any of the other strains. Two strains, Ar 52302 and H 60784 appear to be indistinguishable from each other. Similarly, strains H 60784 and H 60818 are closely related. From the above, the six Orungo virus strains can be classed into three sub-groups (1) the Uganda prototype and Nigerian strain H 13019; (2) strains Ar 52302, H 60784 and H 60818 and (3) strain H 11306. A broader reactivity, showing closer similarity between the different strains was achieved by the constant-virus, varying serum technique.

The production of plaques of same size in Vero cells by the different strains of Orungo virus is a further evidence of

similarities between the different strains. However a plaque reduction neutralization test might be the answer to a definitive differentiation of the Orungo virus strains.

In the survey for N antibodies to Orungo virus in Nigeria, the highest prevalence rate was found in the northern guinea savannah zone, while the lowest rate was found in the rain forest. In contrast, most of Orungo virus isolations in Nigeria were from the rain forest zone which yielded 7 of the nine Orungo virus isolates. This inverse proportion between virus isolations and antibody prevalence rates in the different geographical zone of Nigeria has been observed in previous surveys for Tataguine and Bwamba viruses by Fagbami et. al. (1972), and Tomori et.al. (1974). One explanation is the concentration of virus isolation activities in and around Ibadan and environs in the rainforest zone, where the laboratory is located.

Over 50% of sheep, with about 24% of monkeys and 14% of cows sampled were positive for N antibodies to Orungo virus. Although sheep and monkeys from single locations were studied, the presence of N antibodies in cow sera collected, at Maiduguri, Kano and Ibadan would suggest a widespread infection of the animal population in Nigeria by Orungo virus. However, both further field collections of wild and domestic animals in endemic areas and experimental infection studies are needed to elucidate the epizootiology of Orungo virus in Nigeria.

The isolation of Orungo virus from Aedes spp. and Anopheles spp. in Nigeria and Uganda respectively (Tomori and Fabiyi, op. cit.), and Culex spp. in the Central African Republic (Robin and Sureau op. cit.), would suggest an important role by mosquitoes in the transmission cycle of Orungo virus. Many species of mosquitoes including Aedes, Culex, Anopheles and Mansonia are involved in the transmission of viral diseases between man and animals; for example, Tesselsbron virus (Heymann et. al., 1953), Rift valley virus (Gear et. al., 1955) and Japanese B encephalitis, Scherer et. al. (1959 a, b).

In Nigeria, Aedes spp. may well be active in the natural transmission of Orungo virus between man and animals. In Uganda, only one strain (the prototype) of Orungo virus has yet been isolated. Moreover, serological surveys carried out in Uganda have revealed neither clinical nor subclinical infections in man. It is difficult to explain this striking difference in the epidemiological characteristics of virus strains that are not significantly different from each other. However, a more detailed serological survey in Uganda will probably reveal a truer picture of Orungo virus infection in that country.

At present, there are no clear cut clinical signs associated with Orungo virus by which it can be differentiated from other mild febrile viral infections. In two well documented cases of Orungo

virus infection (Fan lusi et al. op. cit. , Ogunlesi, op. cit.), weakness of the lower extremities which improved with recovery was reported. It may be that Orungo virus infection is associated with the central nervous system involvement. Further field survey with detailed documentation of clinical signs are needed to clearly define Orungo virus infection.

In a serological survey for CF antibodies to arboviruses in Jos, Nigeria, Lawoyin (1974) found 2% of subjects were positive for Orungo virus CF antibodies. Butenko et. al. (1974), in a similar survey carried out in the Kainji Lake area of Nigeria reported over 50% of CF positives for Orungo virus. This high incidence of CF antibodies could result from recent infections by Orungo virus, or persistent infection with subsequent detection of CF antibodies. The high incidence of neutralizing antibodies in humans and domestic animals in Nigeria in addition to the high incidence of CF antibodies show the endemicity of Orungo virus infection in Nigeria. Persistent infection could result from prolonged viremia at was demonstrated with Colorado tick fever (another orbivirus) in man, (Eklund et. al., 1961) and in naturally and experimentally infected animals (Gerloff and Larson, 1959; Du Burgdorfer, 1959, 1960; Burgdofer and Eklund, 1959). Emmons, 1965, 1966, 1967; Emmons and Lennette, 1966, showed that Colorado tick fever virus was associated largely with the erythrocyte

fraction of the blood and showed the value of fluorescent antibody staining to identify virus antigens in blood cells. If the incidence of Orungo virus CF antibody is due to persistent infection in the erythrocyte fraction of the blood, then the presence of virus and virus antigen in erythrocytes for prolonged periods after onset of disease should facilitate the diagnosis by virus isolation or fluorescent antibody staining at any stage of disease. It is significant that during field outbreaks of suspected Orungo virus infections serum samples rather than erythrocytes are employed for virus isolation. This may in part account for the isolation of all human strains of Orungo virus from samples containing erythrocytes, and none from serum samples.

CHAPTER 6

SUMMARY AND CONCLUSION

Orungo virus, a hitherto undescribed virus, originally designated as UgMT 359 virus, was first isolated from a pool of Anopheles mosquitoes caught off human bait in Orungo, Teso District of Uganda in 1959.

It was studied by biochemical, biophysical and sero-immunologic methods to determine its relationship to other viruses and antigenic differences between the different strains of the virus; to delimit the extent of infection with Orungo virus in Nigeria and the host range of the virus.

Orungo virus has a particle size of 63 nm with a core diameter of 34 nm. It has a typical icosahedral construction of the major capsid layer. Virus release from infected cell is by cell lysis.

Orungo virus is labile at 56°C but relatively stable at 37°C and room temperature and also stable to lyophilization. The virus is readily inactivated by ultraviolet irradiation. Orungo virus is only slightly sensitive to lipid solvents, but readily inactivated by betapropiolactone and formalin. It is stable at pH range 5.0 to 7.0, but decidedly labile at pH 3.0. 5-iododeoxyuridine has no significant effect on the multiplication of Orungo virus thus indicating that it may have an RNA core.

Orungo virus was found in all details of morphology and morphogenesis to be similar to other orbiviruses - a group of viruses which are morphologically identical. They share a common property of relative stability to lipid solvents and sodium desoxycholate, libility at pH 3.0 and the lack of antigenic relationship to any of the major serologic group A, B and Buanyamwera.

Laboratory mice, hamsters, rabbits, lambs are susceptible to experimental infection by Orungo virus with resultant viremia or antibody development. Sparrows and day old chicks are not susceptible to experimental infection with Orungo virus. Artificial transmission of Orungo virus by Aedes albopictus and Aede aegypti mosquitoes was demonstrated only on mosquitoes injected with virus by the intrathoracic route.

Orungo virus multiplies with resultant cytopathic effect on Vero cells and BHK-21 cell cultures. Orungo virus also forms plaques in Vero but not BHK-21 cell cultures. No multiplication or CPE was demonstrated after three passages in Aedes albopictus cell line.

Virus strain H60974 previously reported as Orungo virus was found to be Tataguine virus by CF, N and AGD tests. Using these three seroimmunological tests, slight differences were detected in respect of the relation of the Nigerian strains of Orungo virus to the prototype strain isolated in Uganda. By CF strain Ar52302 was

found to show the least relationship to the prototype. Although this difference could not be confirmed by AGD test, where all strains showed identity, on the other hand, by the constant serum varying virus neutralization technique, it was possible to differentiate Orungo virus strains into sub-groups: (1) H13019 and UgMP 359 (prototype). (2) AR52302, H60784 and H60818 and (3) H11306. It is not yet clear if these are artificial differences created by variation in potency of individual strain sucrose-acetone antigen and MAFs. The plaque reduction neutralization tests may yet yield a more definite result. Orungo virus strains show no haemagglutinating activity.

A 23% prevalence rate of Orungo virus neutralizing antibody was found in human sera collected from different parts of Nigeria. The highest prevalence (34%) was found in the northern guinea savannah zone. The prevalence in the other zones were 30%, 25%, and 17% in the southern guinea savannah, derived savannah and the rain forest zones respectively. A high incidence of N antibodies to Orungo virus was detected in sera of sheep, monkeys and cows in Nigeria. Following the isolation of Orungo virus from Aedes spp. mosquitoes in Nigeria, and the demonstration of transmission of the virus by Aedes spp. mosquitoes, these mosquitoes may well be important as biological vectors of Orungo virus between man and animal in Nigeria.

The regularly described symptoms associated with Orungo virus are fever of short duration, headache, myalgia and occasional weakness of the lower extremities which improve with recovery.

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