COMPARATIVE STUDIES ON MOPEIA VIRUSES AND OTHER ARENAVIRIDAE, PARTICULARLY LASSA VIRUS

BY

GRAHAM LLOYD, B.Sc. Hons. (Liverpool), M.Sc. (Manchester), M.I.Biol.

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GLOSSARY

AP	-	alkaline phosphatase
αHBDH	-	alpha-hydroxybutyrate dehydrogenase
BHK	-	baby hamster kidney
BPL	-	beta-propiolactone
BSA	-	bovine serum albumin
BUDR	-	5-bromodeoxyuridine
CAMR	-	Centre for Applied Microbiology and Research
CK	-	creatine kinase
CDC	-	Center for Disease Control (Atlanta)
CFT	-	complement fixation test
CPE	-	cytopathic effect
CV-1	-	green monkey kidney cells
DI	-	defective interfering particles
ΔH	-	energy of inactivation
ΔS	-	entropy of activation
ΔG	-	Gibbs function change
^{o}C	-	degrees centrigrade
EM	-	electron microscopy
FUDR	-	5-fluorodeoxyuracil
γGT	-	gamma glutamyl transpeptidase
GOT	-	glutamine oxaloacetate transaminase
GTNE	-	glycine-tris-sodium-EDTA buffer
GPT	-	glutamine pyruvate transaminase
HAT	-	hypoxanthine-aminopterin-thymidine
HEPA	-	high efficiency particulate air filter
H & E	-	haematoxylin and eosin

IFA	-	immunofluorescent antibody
IgG	-	immunoglobulin class G
IC or i.c.	-	intracerebral
IP or i.p.	-	intraperitoneal
IU/l	-	international units/litre
IUDR	-	5-iododeoxyuridine
IV or i.v.	-	intravenous
K	-	velocity constant
L cells	-	mouse areolar adipose tissue
LCM	-	Lymphocytic choriomeningitis
LAH	-	leucine amino peptidase
LDH	-	lactate dehydrogenase
LGA	-	Lassa (Gwen Aston strain)
LNI	-	$\log_{10}$ neutralizing index
L15	-	Leibovitz medium
M	-	Mopeia virus strain from Mozambique
MOI	-	multiplicity of infection
ND	-	not done
PBS	-	phosphate buffered saline
PEG	-	polyethylene glycol
PME	-	Plaisners minimal essential medium
PTA	-	phosphotungstic acid
RPMI	-	Roswell Park Memorial Institute
s.c.	-	sub-cutaneous
SDS	-	sodium dodecyl sulphate
t'	-	half-life
TCID	-	tissue culture infectious dose
Z	-	Mopeia virus strain from Zimbabwe

G. LLOYD

Comparative studies on Mopeia viruses and other Arenaviridae particularly Lassa virus.

ABSTRACT

Serologically related arenaviruses have been isolated from West Africa, Mozambique, Zimbabwe and the Central African Republic. Human disease is only associated with the West African isolates. The virulence of Mozambique, Zimbabwe and Central African Republic isolates in humans is not known.

This Thesis is an account of work carried out by the author to compare the biological characteristics of isolates from West Africa, Mozambique and Zimbabwe. It describes the successful isolation and identification of the aetiological agents, their physicochemical and antigenic characteristics and describes in vivo studies using mice, guinea pigs and Rhesus monkeys.

A direct comparison was made with a patient diagnosed as having Lassa fever. The disease in man and monkeys following infection with Lassa virus was similar. The Rhesus monkey and guinea pig proved suitable experimental models in which to study and compare the pathogenic responses and also to evaluate various aspects of protection. These animal models when immunised with the viruses from Mozambique and Zimbabwe were protected when subsequently challenged with Lassa virus.

The Mozambique and Zimbabwe isolates proved to have morphological and physicochemical characteristics not dissimilar from West African Lassa viruses and those members of the arenavirus family from South America. Serological and immunochemical investigations suggest the existence of both common and unique antigenic determinants on the viruses from Mozambique, Zimbabwe and West Africa. This grouping also coincides with the geographic separation of the viruses, i.e. Lassa - West Africa and Mopeia - south east Africa. Similar differences in host susceptibility have also been demonstrated. Lassa virus produces a fatal haemorrhagic disease while Mopeia isolates produce only an asymptomatic infection. The combined data suggests the possibility of two virus groups within the 'Old World' arenavirus classification. The proposed name, 'Mopeia', forms one group and includes the viruses from Mozambique and Zimbabwe. The Lassa strains from West Africa form the second group.

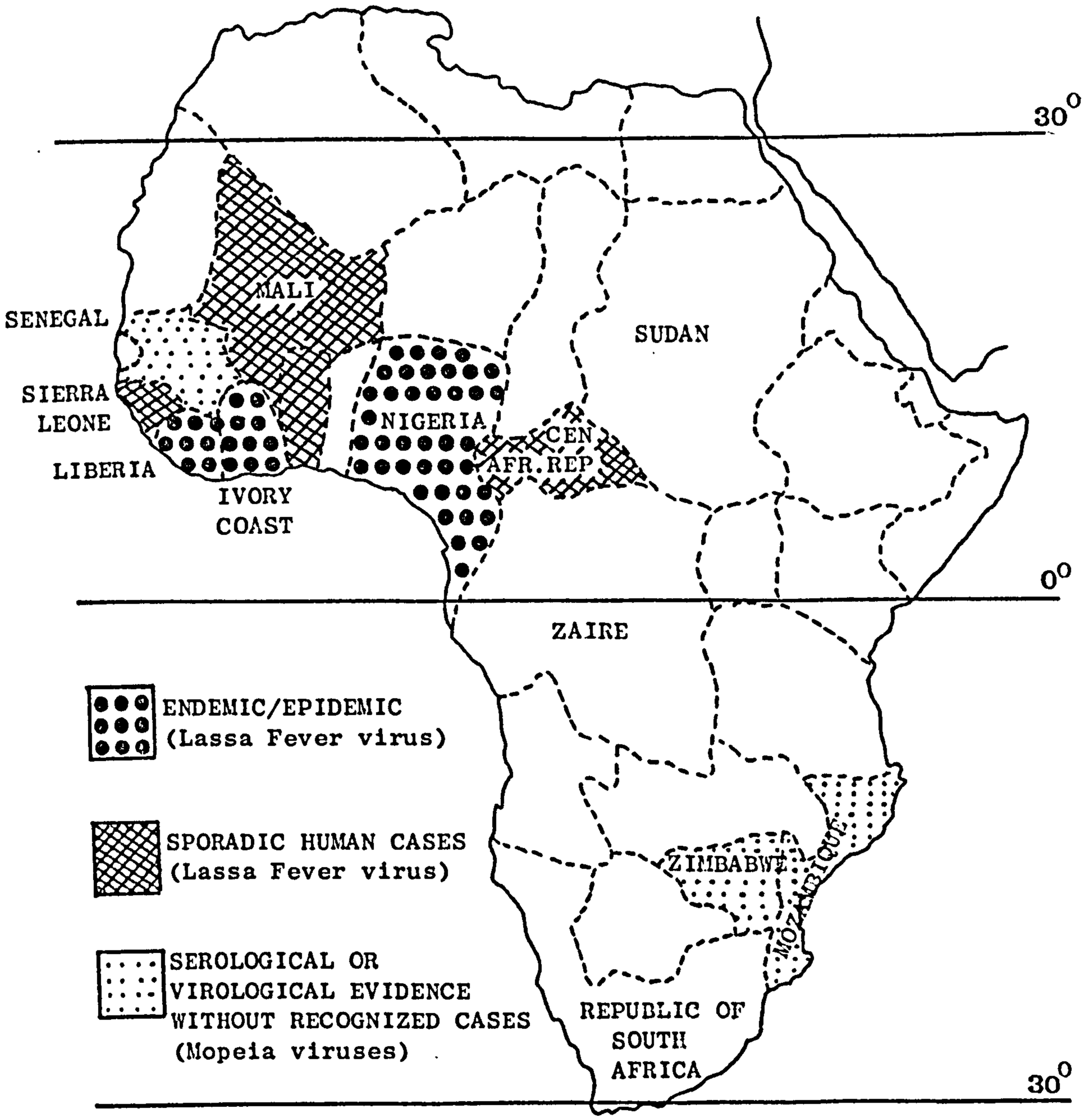
It is suggested that the Mopeia viruses are minor antigenic variants of Lassa and should be included within the arenavirus family.

1. INTRODUCTION AND HISTORICAL BACKGROUND

The arenavirus taxon takes its name from the sand-sprinkled (arenous) appearance when viewed by the electron microscope. They are a family (arenaviridae) of pleomorphic, enveloped viruses containing a single-stranded RNA genome of molecular weight of about 4×10^6 (Ramsingh et al. 1980) in two segments of negative polarity. The first recognised member of the group, Lymphocytic choriomeningitis (LCM), is considered to be the prototype. The group is divided (Fig. 1) into the LCM complex ('Old World') and the Tacaribe complex ('New World') (Fig. 2) on the basis of immunological cross-reactivity as determined by complement fixation or immunofluorescent staining.

Nearly all the 12 members (Figs. 1 & 2) so far described cause acute or persistent infections of rodents. LCM has been implicated in cases of aseptic meningitis in man since its original description (Armstrong and Lillie, 1934) although these have been largely laboratory-acquired infections. Three arenaviruses - Junin (Parodi et al., 1958), Machupo (Johnson et al., 1965), Lassa (Buckley and Casals, 1970) - cause severe haemorrhagic diseases in man in Argentina, Bolivia and West Africa respectively. They represent a serious public health problem in these countries. Lassa in particular has achieved public notoriety owing to its association with severe febrile illness among missionaries and travellers returning from rural parts of West Africa. Human infection of man with LCM is rarely fatal whereas the mortality rate of the haemorrhagic arenaviruses ranges from 10 to 50% in individual outbreaks.

Two major rodent families (Table 1) are associated with arenaviruses. They are Muridae (mice, rats) and Cricetidae (voles, lemmings, gerbils). Lassa, LCM and the more recent isolates of Mopeia from Mozambique and Zimbabwe are all found in members of the Muridae family. The 'New World' viruses are confined to Cricetine rodents whose members frequent



N.B. LCM (Worldwide)

Fig. 1 Geographic Distribution of Lassa-type Viruses in Africa

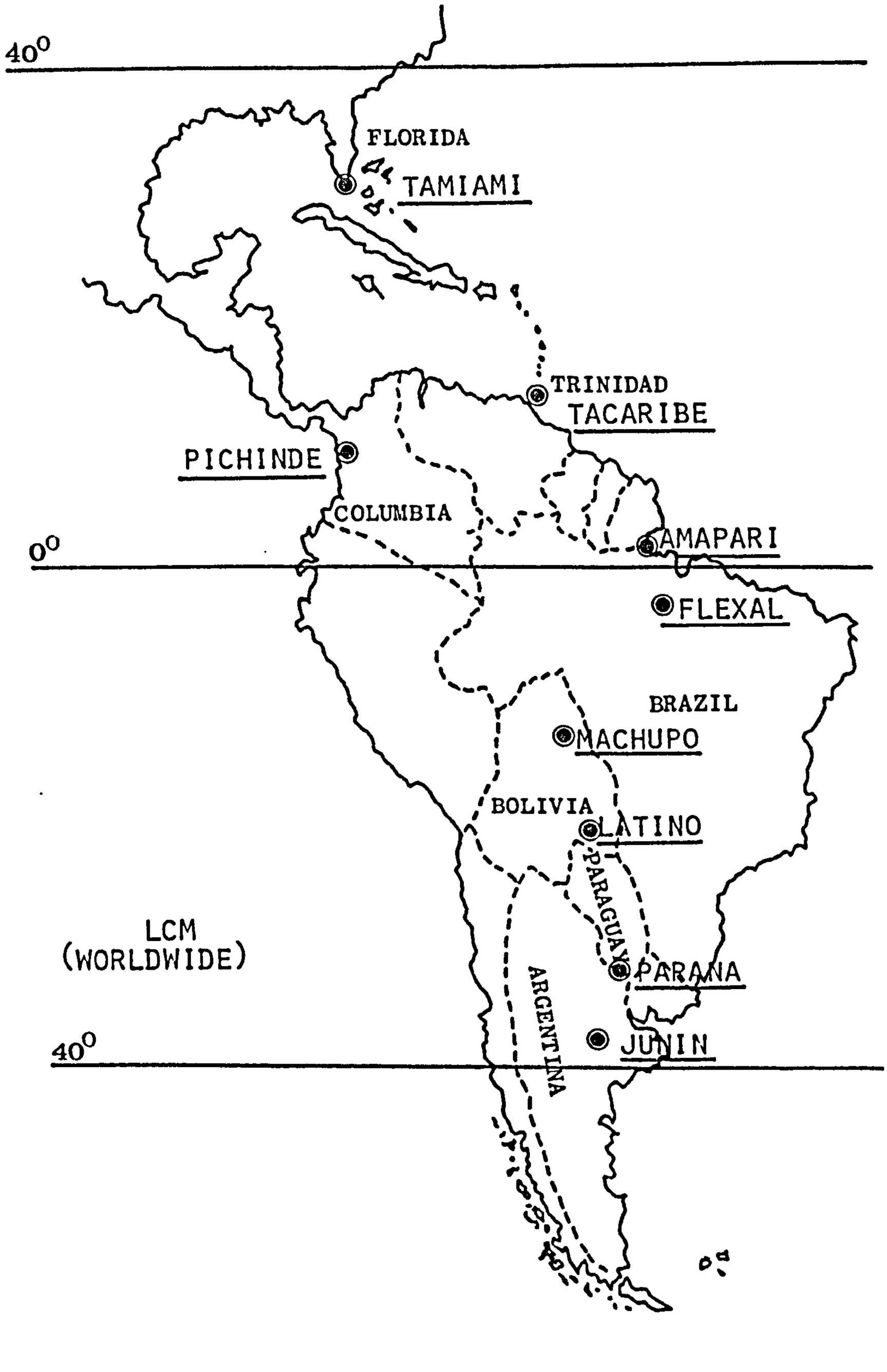


Fig.2 Approximate Distribution of Arenaviruses in South America

open grasslands and forests. The exception is Tacaribe virus, which was originally isolated from the fruit bat, Artibeus literatus, in Trinidad in 1956-1958 and has not been isolated since (Downs et al. 1963). Tamiami virus which was isolated in Florida, USA from the cotton rat, Sigmodon hispidus, completes the so far characterized Tacaribe complex of arenaviruses (Calisher et al. 1970).

TABLE 1. PRINCIPAL NATURAL VERTEBRATE HOSTS OF ARENAVIRUSES

Virus	Vertebrate	Habitat
Tacaribe	Artibeus lituratus	Tropical forest
	Artibeus jamaicensis	"
Junin	Calomys laucha	Hedgerows, farm fields
	Calomys musculinus	"
	Akalo azarae	"
Machupo	Calomys callosus	Grassland, forest, farm fields, houses
Amapari	Oryzomys goeldi	Tropical wet forest
	Neucomys guianae	" " "
Tamiami	Sigmodon hispidus	Grassland, early secondary forest
Pichinde	Oryzomys albigularis	Tropical cloud forest
Parana	Oryzomys buccinatus	Tropical dry forest
Latino	Calomys callosus	Grassland, forest edge, farm fields, houses
LCM	Mus musculus	" "
Lassa	Mastomys natalensis	" "
Mopeia	" "	" "
Flexal	Neocomys spp.	" "

LCM virus remained unclassified until shortly after the morphology was established in 1968 (Murphy et al. 1975). The first obvious link between LCM and other viruses came in 1964 when it was found that infection of neonatal rodents, Calomys species, with Machupo virus

resulted in life long viraemia in the absence of specific neutralizing antibodies (Johnson et al. 1965; Johnson et al. 1975; Webb et al. 1967). Since the same response was known to exist with the murine LCM system, serological and morphological comparisons followed (Hotchin 1971; Lehmann-Grube, 1979) . Machupo and all the viruses with which it shared complement fixing antigens (Tacaribe complex) were antigenically and structurally related to LCM (Murphy 1975; Rowe et al.1970a). This led to the proposal and definition of a new virus group - arenovirus (Rowe et al., 1970b). To avoid possible confusion with Adenovirus (especially in oral conversation) the International Committee on Nomenclature of Viruses (ICTV) 1970 chose the name, arenaviruses, to describe the genus. Family status (arenaviridae) by the ICTV was confirmed in 1975 (Fenner 1976).

With the discovery of Arenavirus-like particles (Mopeia) in southern Africa, interest was directed towards studying their relationship to Lassa fever and establishing the significance of this virus within the African continent. Preliminary investigations demonstrated an antigenic link between both viruses but also indicated that the viruses differed significantly in their expression of virulence for man. These observations parallel those found with the Arenaviruses of South America. Lassa fever is perhaps the most publicised of all the viral haemorrhagic fevers with a case fatality rate of 36-67% among hospitalized patients. Twenty one medical workers are known to have been infected, of whom 10 have died. Once Lassa has been successfully transmitted from its natural reservoir host to man, it is capable of adaption to produce man-to-man transmission. It is theoretically possible for such an infection to be introduced from its country of origin to countries where the natural host does not exist and still be capable of successful transmission. Hence the worldwide public health concern.

Lassa virus was first isolated from an American missionary nurse (Fig.3) following the sequential infection of two other nurses in Nigeria

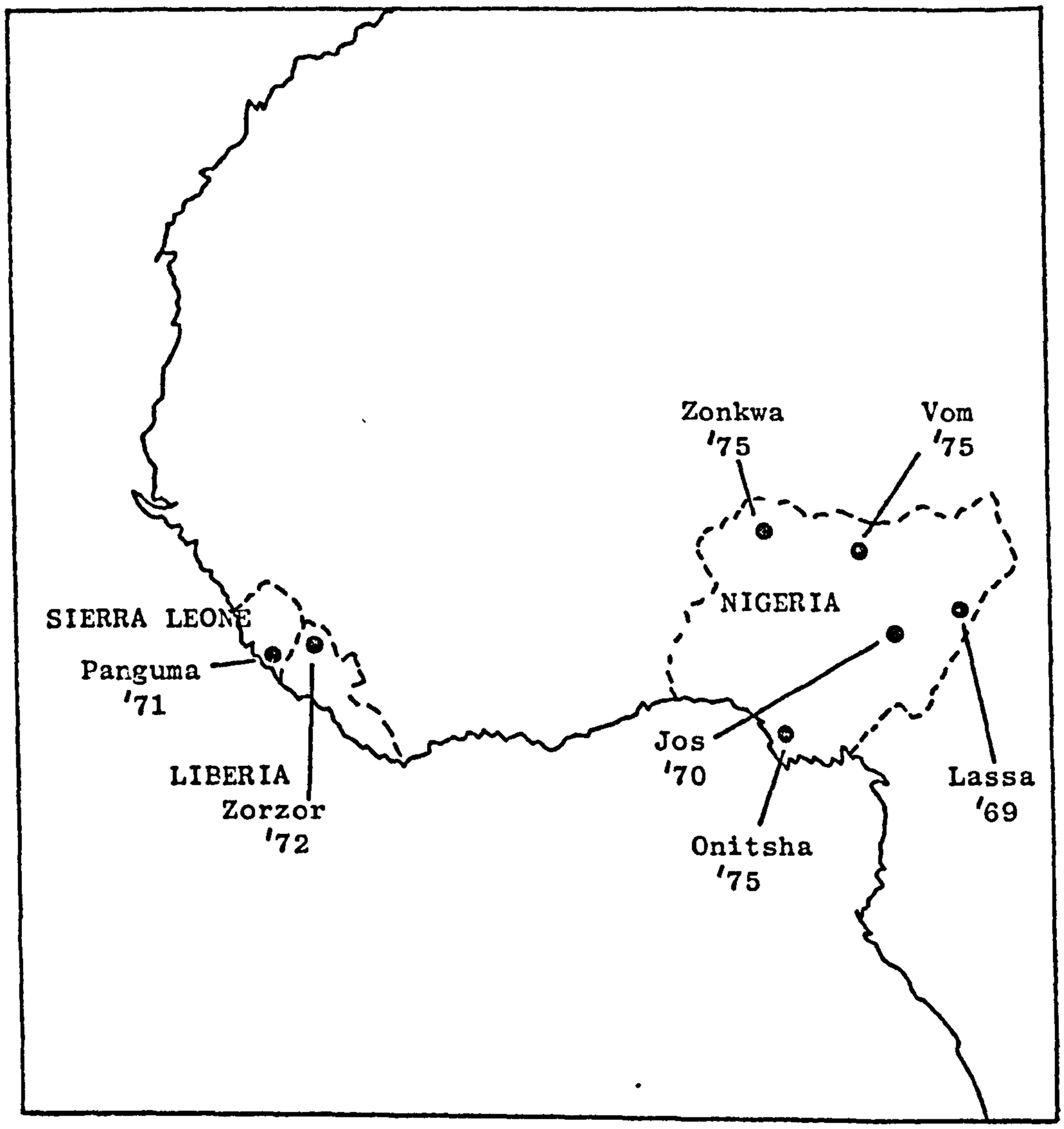


Fig.3 Outbreaks of Lassa Fever in West Africa from 1969

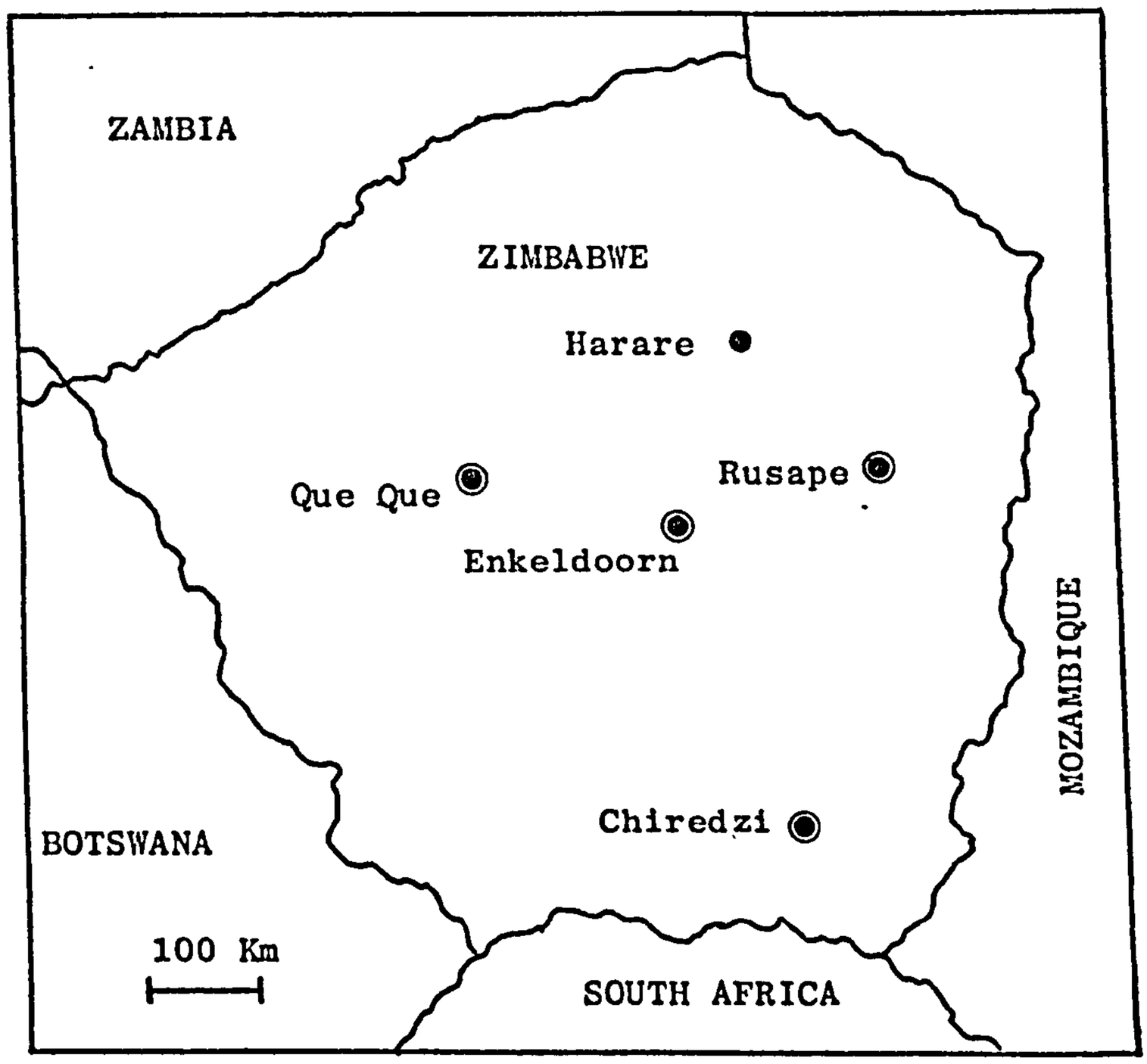


Fig.4 Map of Zimbabwe, showing areas where rodents were captured for detection of Arenavirus infection. Capital city, Harare, is indicated as a reference point.

(Frame et al. 1970). The virologist who made the isolation in turn became infected. A seasonal outbreak of Lassa fever with a high mortality of 53 per cent. among 23 patients confined to hospital was reported in Jos, Nigeria in 1970 (White 1972). In March 1972 further cases of Lassa fever occurred among 4 patients and 7 members of staff in a hospital in the Zorzor district of Liberia, and 4 patients died (Monath et al., 1973). The fourth major outbreak was not hospital-associated and took place between October 1972 and October 1974 in and around Panguma township, Sierra Leone. There were 64 cases, most of whom acquired their infection in the community. Several family outbreaks occurred. Between January 1969 and July 1978 there have been 17 reported outbreaks involving 386 cases with 105 deaths, an overall case-fatality rate of 27.2% (Galbraith et al., 1978). Previously estimated fatality rates for Lassa fever were mainly based on hospitalized cases; when serological findings are taken into account, the case fatality-rate in the community was calculated to be less than 5%. Since 1978, Lassa fever has continued to occur in West Africa, but usually as sporadic cases. Quite often medical personnel are those who manifest the disease, presumably being infected through contact with a febrile patient. In an endemic area of Lassa virus activity, it has been found that almost half of the patients presenting a febrile illness have had a Lassa infection but very few develop severe disease.

Lassa virus is present in several West African countries although outbreaks are confined to Nigeria, Liberia and Sierra Leone. Major studies on the epidemiology of the disease have shown Lassa to be a zoonosis transmitted to man from a rodent reservoir, known to be Mastomys natalensis, widely distributed throughout Africa (Demartini et al. 1975). The disease is endemic in certain areas of West Africa, particularly Sierra Leone, Liberia and the Jos plateau of Nigeria, while other areas of Nigeria together with Guinea, Senegal, Mali, Central African Republic and Zaire have been implicated on historical or serological evidence (Monath 1975).

The mode of transmission of Lassa virus from rodent to man or from man-to-man is not yet known. Similarly the pathogenesis of Lassa virus infection in its natural host is still not fully understood. Like other arenaviruses, Lassa produces a persistent tolerant infection in its rodent reservoir host, with no apparent ill effects. The animals are infected at birth and remain infective during their life-time, excreting the virus in urine and other body fluids. Transmission to man probably occurs when he comes in close contact with rodents, and could be either via the respiratory or the gastrointestinal route from the consumption of contaminated food or water (Monath, 1975). Monath suggested that the low level of sanitation, the storage of grain and food within houses and the ease with which rodents infect mud and thatch houses enhance the contact between rodents and man. The means by which the virus is spread from person-to-person is still not clear. Medical attendants or relatives providing direct personal care are most likely to contract the infection. Lassa virus has been isolated from the blood, pharynx and urine of patients, suggesting that indirect airborne spread of the virus as well as mechanical transmission are most likely (Buckley and Casals, 1970) . Community infection in the endemic areas is dominated by the pattern of rodent-to-man transmission . Fraser et al. (1974) suggested that the clustering of sero-positives among people who shared the sleeping accommodation of the cases, might be due to person-to-person transmission possibly by the respiratory route. This possibility cannot be proved or disproved on epidemiological grounds. If it does occur then it is considered of little importance in the natural history of the disease pattern.

In the hospital situation the reverse is true. Here rodent-to-man transmission may be discounted and the only possible mechanism is person-to-person spread. This occurs under three conditions: 1. Direct inoculation of the virus through the skin, 2. Nosocomial transmission as a result of

close patient contact, 3. Airborne transmission through droplet material derived from the respiratory tract of the patient. Each emphasises the need for extreme caution within a research programme including Lassa viruses. Of the 17 recorded episodes, 11 were hospital outbreaks involving a total of 57 cases with 25 deaths (43.8%). Two were laboratory infections and two were prolonged community outbreaks studied over a period of 2-3 years respectively (Fraser et al., 1974, Keane and Gilles 1977). The observed case-fatality case of 43.8% among nosocomial (hospital-acquired) infections is a sharp contrast to the rate of 24.3% in community acquired infections.

Information about the ecology of the Lassa infections is far from complete. The evidence so far suggests that the natural reservoir of the multimammate rat, Mastomys natalensis which has been the only animal carrier identified among 26 species tested in Sierra Leone and Nigeria. The numbers of animals tested so far have been small and their geographical area limited. While there can be no doubt that this species acts as a reservoir, some epidemiologists believe there is insufficient evidence to identify it positively as the primary reservoir of the virus. Whatever may prove to be the primary source, the function of Mastomys as a link reservoir and its close association with residential compounds in endemic areas, make it the most likely intermediate source of infection for the majority of human cases. What is of concern results from the ecological threat which could occur from the introduction of the virus into a virgin community such as the United Kingdom, Europe and North America.

In contrast, Mopeia virus has been isolated from the same rodent species (Mastomys) in Mozambique and Zimbabwe where haemorrhagic fever disease like that produced by Lassa virus has not been reported. During studies on arbovirus infections in Mopeia, Central Mozambique, five

virus strains were isolated from the spleens of Mastomys natalensis which showed no immunological relationship to any known arbovirus (Wulff et al. 1977). The agents isolated from this study suggested that they were morphologically and immunologically related to Lassa. Four of 19 sera from Mastomys captured in the study area had antibodies to both Lassa virus and one of the unidentified strains. Although not definite, the differences noted in results from complement fixation and indirect immunofluorescent tests suggested that the viruses from south east Africa were not identical to the West African Lassa virus. In the second study, six strains of Mopeia virus were isolated from Zimbabwe, and this was the first study to suggest that these agents were more widely distributed than previously recognized (Johnson et al., 1981). Rodents were captured from four areas in central, eastern and southern Zimbabwe (Fig.4), Chiredzi; Rusape; Enkeldoom and Que Que. Upon capture, rodents were bled by heart puncture and liver and spleen tissue removed. The study also included the differential identification of rodent species in an attempt to determine a relationship between the isolates and specific rodent species. Immunofluorescent antibodies to Mopeia were found in 11 of 55 Mastomys natalensis and one of 13 Aethomys chrysophilus rodents captured near Que Que and Chiredzi, Zimbabwe. All Mastomys having virus or antibodies to Mopeia were of the chromosomal form 2N = 32 (M. pramomys natalensis), none being found in the other member of the Mastomys group M. coucha (chromosomal type 2N = 36).

In terms of host-parasite relationships, data relating to Mopeia virus provide a strong stimulus to answer a variety of questions. As antibody had been found in the species A. chrysophilus, their role in the natural maintenance of Mopeia virus has to be resolved. A second area of study needs to elucidate the specificity of Mopeia to M. natalensis (2N = 32) in southern Africa. In order that M. coucha (2N = 36) could be disregarded as

a reservoir a large number of rodents must be tested. M. coucha and M. natalensis differ in their distribution throughout southern Africa (Gordon, 1978; Green et al. 1978; Green et al., 1980). Any difference in their susceptibility to Lassa and Mopeia could have important implications for related human diseases. No evidence as to the extent of Mopeia virus infections and antibody levels in the human population is at present known.

Since Lassa virus appears to be a single-host rodent parasite, and because M. natalensis is widely distributed, the presence of Lassa virus or a closely related arenavirus in south-east Africa does not represent a biological surprise (Isaacson, 1975) . Since Mopeia virus was found more than 2,500 miles from the place in which Lassa is endemic, it is of special importance to determine whether the new strains are antigenically related, pathogenic for man and whether M. natalensis of West Africa and south-east Africa are truly identical.

Since structurally and morphologically the arenaviruses appear to be a homogeneous group, extensive studies have been directed at determining their molecular biological and physical properties. The major component of arenavirus particles (including Lassa) is the nucleocapsid protein with a molecular weight ranging from 54,000 - 68,000 (Clegg and Lloyd, 1982; Howard and Simpson, 1980; Pederson 1979). Lassa virus also has two envelope glycoproteins with molecular weights similar to those found in most other members of the family (Howard and Simpson, 1980) although Tacaribe and Tamiami viruses are reported to have only a single envelope glycoprotein (Gard et al., 1977). Other minor proteins have been found in purified arenavirus preparations, but their status as true components of the virion is at present unclear. Thus, the molecular biochemical structure has demonstrated limited differentiation between the 'Old' and 'New' World arenaviruses.

Alternatively, arenaviruses are known to show varying degrees of antigenic relationships using complement fixation and immunofluorescent

antibody tests. Cross-reactivity among the arenaviruses using complement-fixation tests (CFT) demonstrates a close association between Amapari, Junin, Machupo and Tacaribe viruses (Casals et al., 1975). LCM antisera was also reported to show a cross-reactivity with other members of the family with the exception of Lassa. Evaluation of reciprocal antibody titres for at least one representative of each known arenavirus serotype, leads to some conclusions concerning their immunofluorescent antibody (IFA) inter-relationship. Two groups can clearly be distinguished. The first comprises the 'Old' World arenaviruses, i.e. Lassa and LCM, both viruses cross-reacting at a very low level with other members of the arenavirus group. Although originally isolated in North America, LCM virus is considered an 'Old' World agent because its rodent reservoir Mus musculus, is believed to have originated in Asia. Antisera obtained from man and animals against 'New' World arenaviruses, which forms the basis of the second group, showed strong cross-reactions with most antigens except Lassa and LCM (Wulff et al., 1978)

To date, there has not been any reliable data produced on arenavirus cross-neutralization. Difficulties in demonstrating convincing specific neutralization of arenaviruses tends to preclude a final judgment on their identity. Casals (1977) claimed that members of the Tacaribe complex do not cross-react in neutralization assays. The only evidence to date of any cross-neutralization between arenaviruses is with Tacaribe-immunized guinea pigs which also produce anti-Junin neutralization antibodies (Weissenbacher et al., 1976; Coto et al., 1980).

The foundation of this thesis is a study of the biological characteristics of Mopeia viruses and other arenaviridae, particularly Lassa virus. This involved comparison of their morphological, physico-chemical,molecular biological, antigenic and pathogenic properties using in vitro and in vivo models. It is proposed on this basis that Mopeia viruses

are minor antigenic variants of Lassa virus and the aim of the thesis is to examine this hypothesis critically with a view to their inclusion in the arenavirus family.

2. METHODS AND MATERIALS

2.1 CATEGORY A FACILITIES

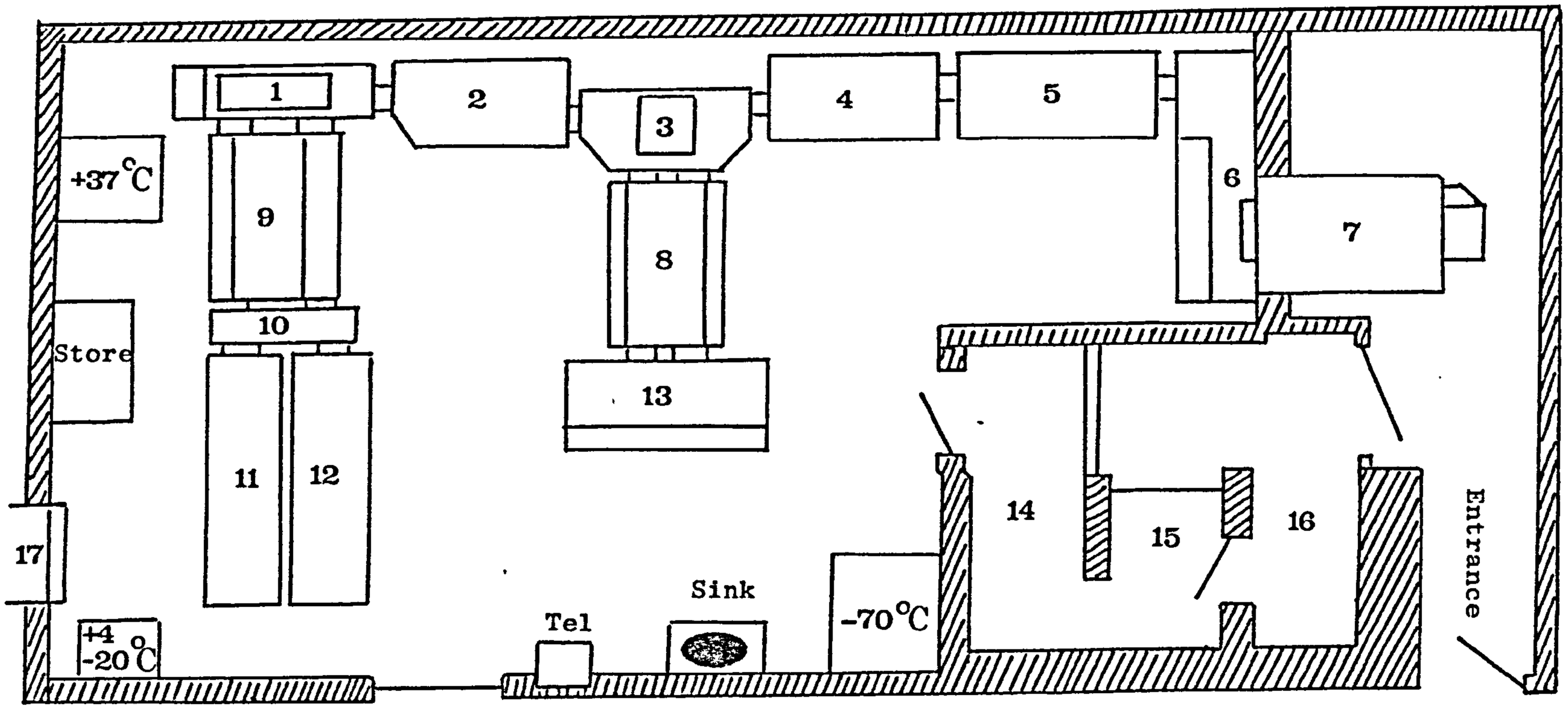
2.1.1. Laboratory Suite

Since Lassa virus is categorized a Category A pathogen, all experiments involving this agent were carried out under the most stringent precautions to prevent infection of laboratory personnel. All serology and tissue culture studies were carried out in the maximum security laboratory at the Centre for Applied Microbiology and Research.

The unit (Fig.5) consists of a laboratory connected to a changing room with shower and an annex housing the clean side of a double-doored autoclave. All three rooms operate under a gradient of negative pressure and are ventilated by a plenum and exhaust system. The input air is filtered to remove coarse particles and the exhaust air is passed through High Efficiency Particulate Air (HEPA) filters in the ceiling. Entry to the unit is gained through the autoclave annex to the changing room where everyday clothing is exchanged for protective clothing. This, briefly, consists of underpants, pyjamas, socks, gown and surgical boots. On entering the laboratory the staff put on two pairs of gloves, a thin latex pair and a pair of white linen ones.

The laboratory consists of a series of inter-connecting gas tight aluminium safety cabinets which operate under negative pressure (Figs. 6 - 11), each cabinet having gauntlet covered portholes for access. The negative pressure is provided by two banks of three fans; each fan being capable of pulling approximately (510m^3/h) of air through the system, giving a flow rate of between (60m^3/h) of air through each cabinet. Under normal operative conditions only a single fan is used from one of the banks. The others are mainly back-up fans. If there is a need, the negative

FIG.5 HIGH CONTAINMENT SUITE CAMR PORTON



1. Dunk & Pass Box
2. Incubator Cabinet
3. Pass Box
4. Work Area
5. Fridge & Centrifuge Cabinet
6. Autoclave Cabinet
7. Autoclave
8. Work Area
9. Work Area
10. Pass Box
11. Animal Cabinet
12. Incubator Cabinet & Inverted Microscope
13. I.F. Microscope Cabinet
14. Dirty Changing Room
15. Shower
16. Clean Changing Room
17. Room Autoclave
18. Emergency Exit

pressure in the system can be increased by a second or even a third fan which would increase the flow rate to approximately ($170m^3/h$). In the case of a power supply failure, the Establishment's stand-by generator comes into operation in well under a minute. If this too should fail, as soon as the negative pressure in the system drops to 0.2 inches of water pressure, an automatic pressure switch situated in the trunking switches on 2 battery operated fans which are capable of maintaining this negative pressure for at least 24 hours.

Air is filtered into and out of each of the cabinets and is again filtered before it leaves the trunking at the end of the system. Material can be passed into the system through a double-ended autoclave, the autoclave acting as an air-lock. There is a safety device to ensure that both doors cannot be opened at the same time. Material is then passed along from cabinet to cabinet through inter-connecting portholes.

Within the cabinet system are housed a small centrifuge (MSE Minor), built-in $+4^{\circ}C$ refrigerator and $37^{\circ}C$ incubator, 2 water baths, one operating at $37^{\circ}C$ and the other at $60^{\circ}C$; one inverted microscope (Prior), one multipurpose microscope (AO Microstar) predominantly used for fluorescence and a Flow 'Titertek' automatic E.L.I.S.A. analyser. Specimens are brought out of the system for storage purposes through a dunk tank containing 5 per cent. hypochlorite solution, following established codes of practice. All material which is discarded within the system is placed in autoclavable bags and decontaminated in the autoclave, access to which is gained directly from the cabinet system. Each cabinet is decontaminated after working with formaldehyde vapour overnight.

In the laboratory itself, there are $-70^{\circ}C$, $-20^{\circ}C$ and $+4^{\circ}C$ refrigerator units, a $37^{\circ}C$ incubator and a store cupboard for everyday laboratory equipment. There is a second autoclave leading from the laboratory itself. This is mainly used for the sterilization of clothing plus other items that require autoclaving from the room.

Figs. 6 to 11 show the cabinet layout in the Maximum Containment Laboratory at the Centre for Applied Microbiology and Research

Fig. 6. Cabinets housing +4°C refrigerator (▲), bench centrifuge and double-ended autoclave.

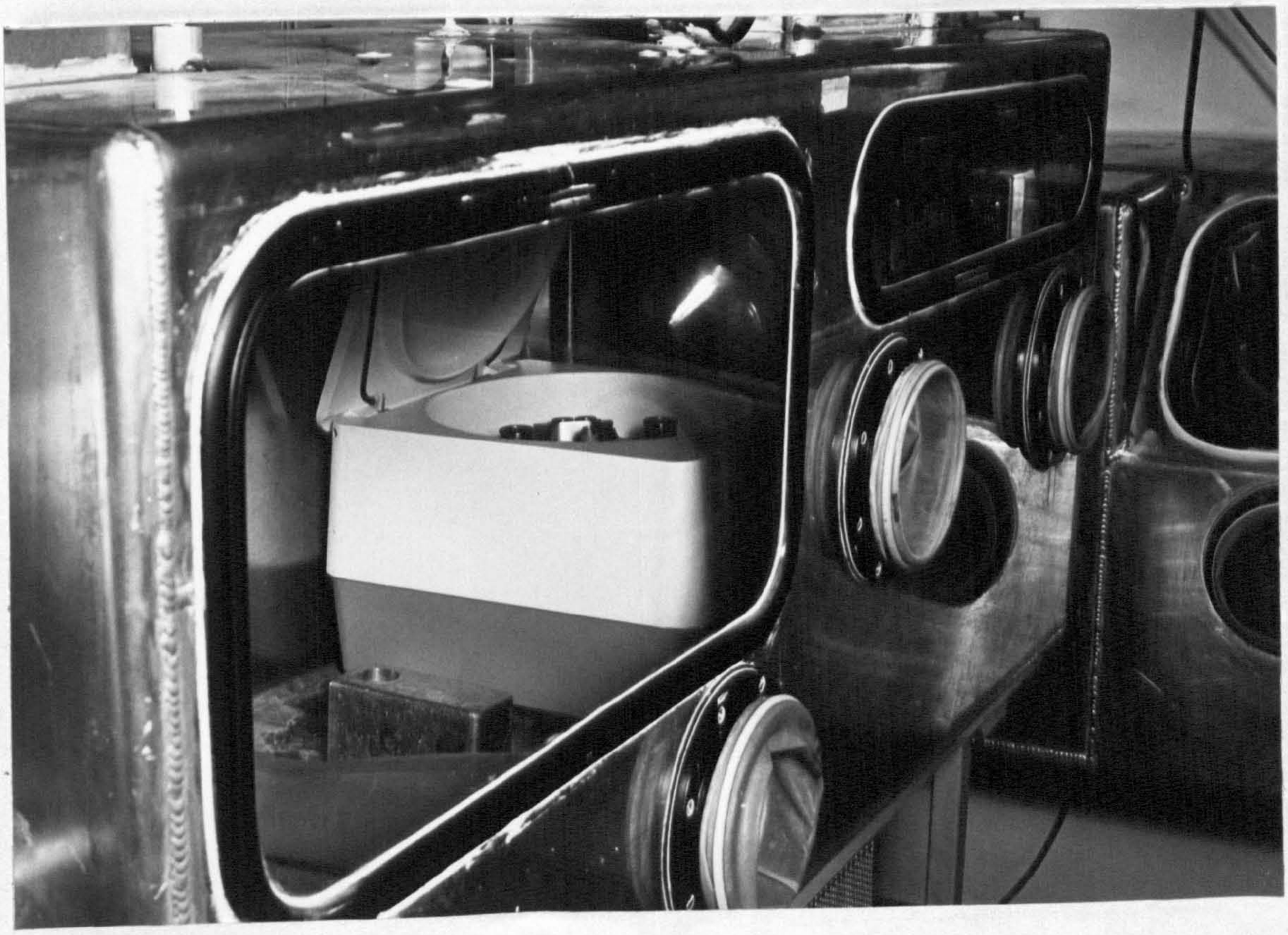


Fig. 7. Cabinet housing bench centrifuge.

Fig. 8. Cabinet containing two 37°C incubators.

Fig. 9. Double sided working cabinets.

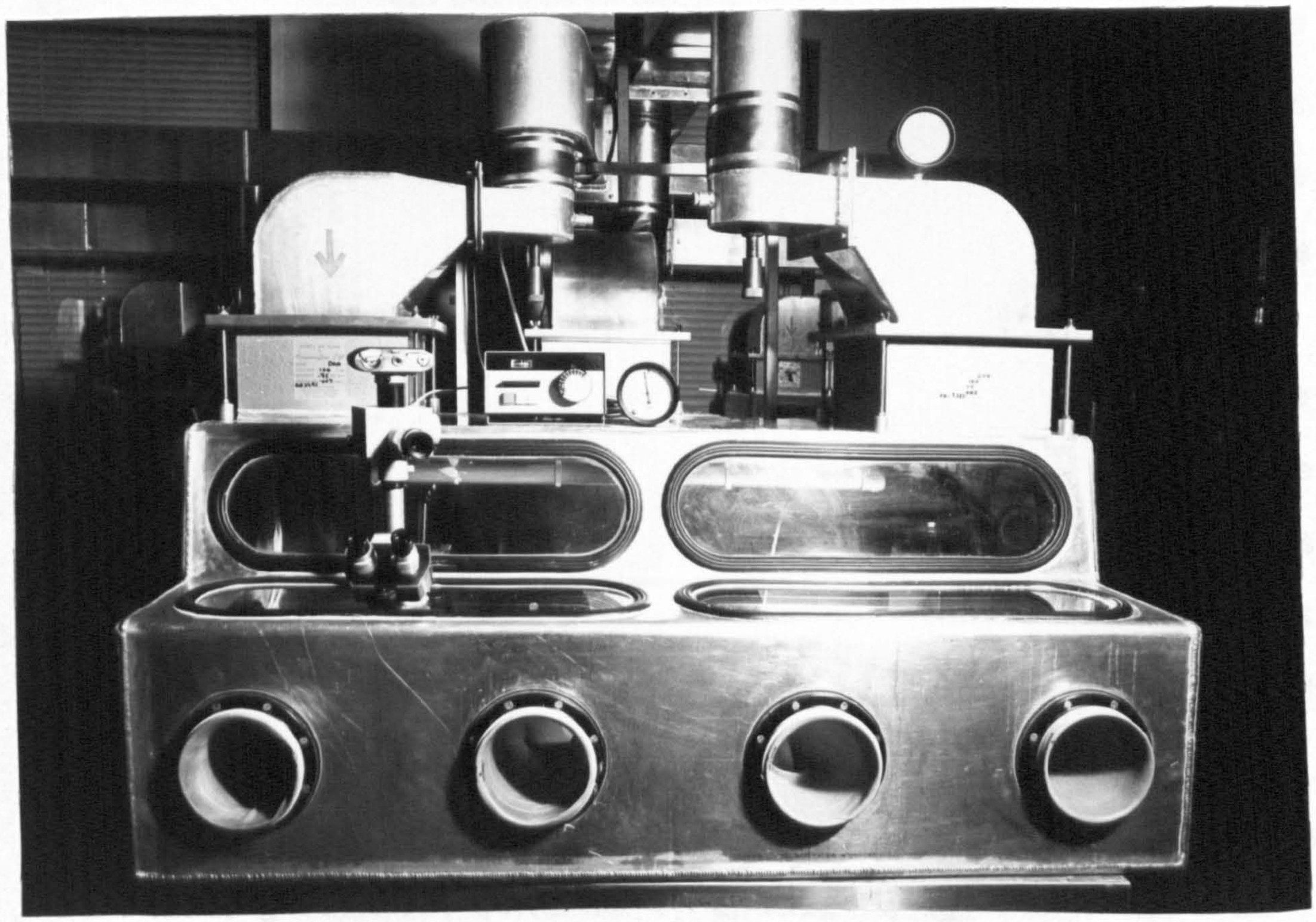


Fig. 10. Cabinet designed around an immunofluorescent microscope. The light source, power packs and main body of the microscope are sealed inside the cabinet from the eye-piece head and camera which are positioned on the outside.

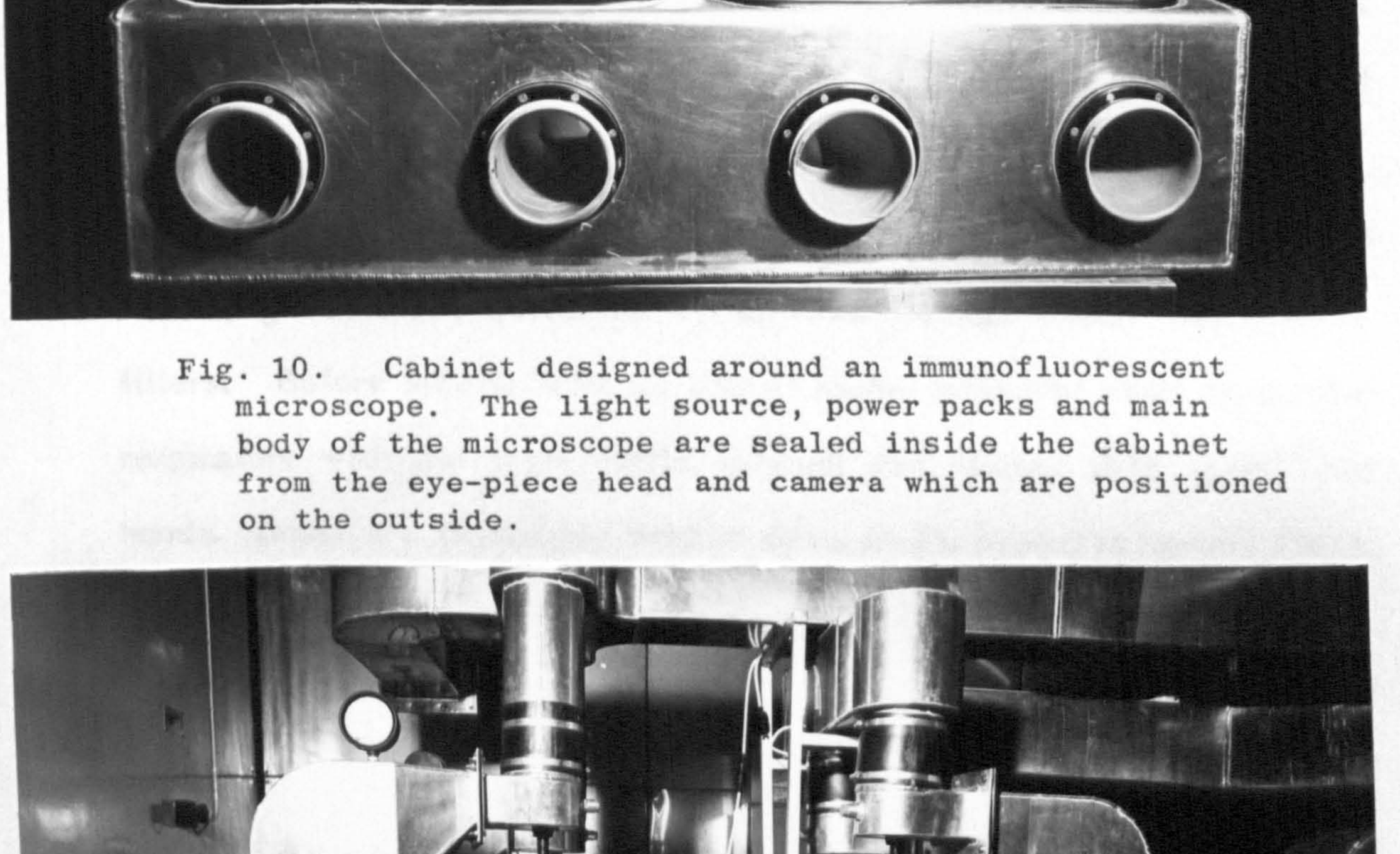


Fig. 11. Cabinet containing two 37°C incubators and inverted light microscope.

2.1.2. Animal Laboratory

Animal experiments, ultracentrifugation, growth of large batches of virus and biochemical analysis were carried out in a suite of rooms with entry and exit through an airlock and shower. Personnel wore protective clothing, gloves, rubber boots and full-face biological respirators and hoods (Figs. 12 & 13).

This suite of rooms has a gradient negative pressure from corridor to suite. Entry can only be gained through the shower and air-lock. At the opposite end of the animal suite a double-ended autoclave is housed. This is large enough to use as an airlock for transport of animals and equipment into the suite. Animal bedding, waste, post-mortem material are all autoclaved before removing to the clean side of the autoclave.

All effluent from this suite of rooms is sterilised by boiling in special collecting tanks and extracted air filtered through double HEPA room filters. Before leaving infected animal rooms personnel swab down their respirators with 5% hypochlorite solution and discard their gowns and hoods. Boots are thoroughly washed down in 5% hypochlorite and rubber gloves removed and left to soak in the hypochlorite solution. Having left the infected rooms, the remaining clothing is removed in the air lock and discarded. Boots and respirators are stored in lockers. Personnel shower completely before returning to the clean changing area.

2.2 VIRUSES

2.2.1. Lassa strain (LGA 391)

The origin of this virus was Zaria, Nigeria and it was isolated at Porton in November 1977. The patient (G.A.) was a part-time doctor in a paediatric ward at Ulussa Hospital. Her symptoms started on 16 October 1977 when she was admitted to hospital. Lassa virus was isolated from her blood which was taken on 25 October. The isolation was carried out in vero cell cultures and confirmatory diagnosis carried out by immunofluorescence using known anti-Lassa serum.

Fig. 12. Cabinet containing LKB (Ultralab) analyser, dilution and analytical data microprocessor for serum biochemistry studies.

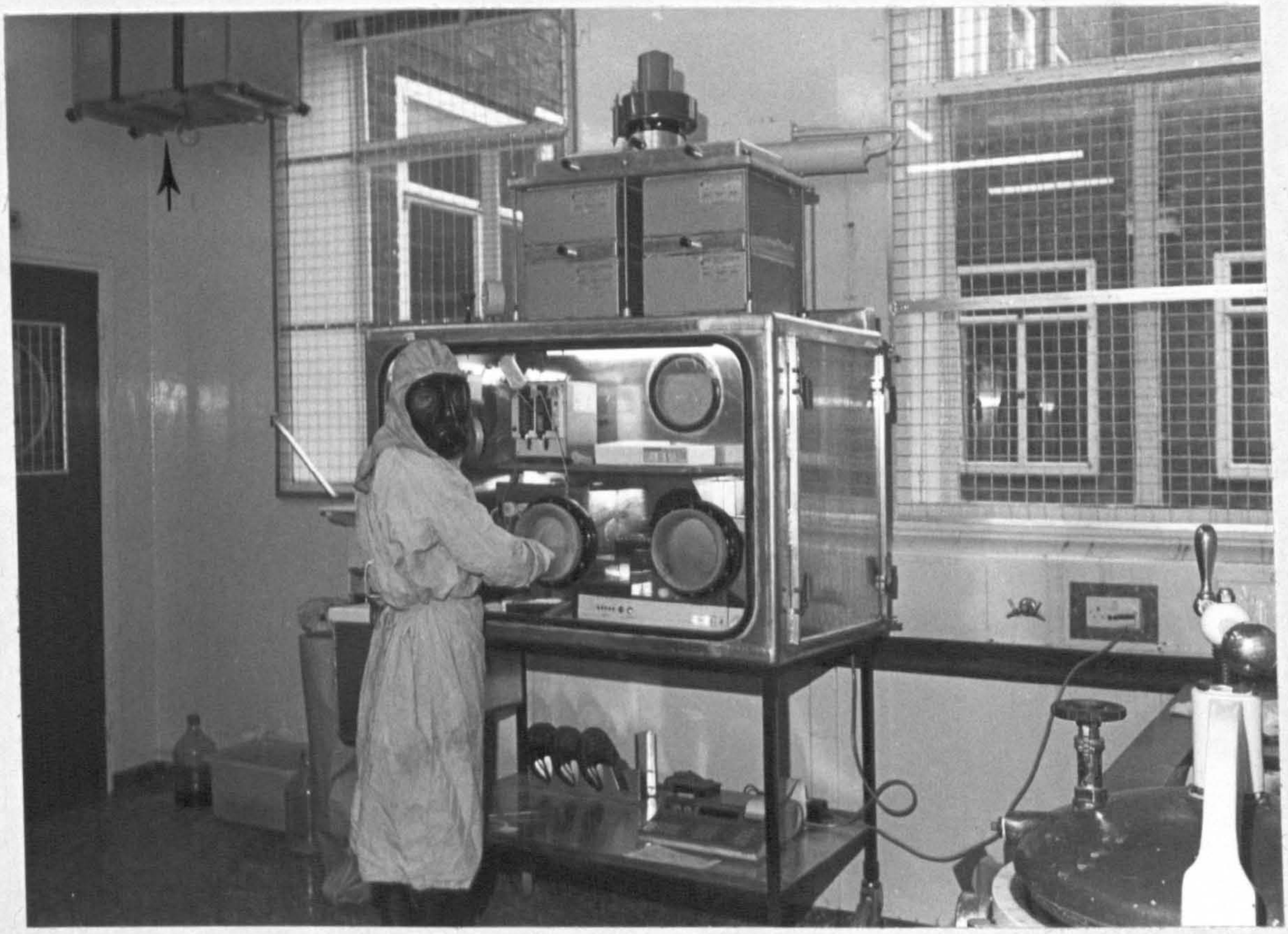


Fig. 13. This shows the clinical biochemical cabinet positioned in a Category A animal room, together with the protective clothing used by personnel. Room filtration is through double HEPA filter in the ceiling (arrow).

For the purposes of the studies in this thesis the original LGA 391 strain was used and had been passed three times in Vero cultures. A stock of this pass 3 material was made and stored in 0.2 ml aliquots at -70°. The titre of the stock was estimated to be $10^{7.8}$ tissue culture infectious $doses_{50}(TCID_{50})$/ml. Later stocks were made using roller bottles the virus titre of which was raised to 10^9 $TCID_{50}$/ml.

TABLE 2. LASSA AND MOPEIA VIRUSES STUDIED

Virus	Reference	History	Source	Laboratory History
LGA391	Grundy et al, (1980)	Human case, originated in Nigeria, hospitalised in UK	Serum	Isolated in Vero cells, CAMR passed x 3
M20410	Wulff et al. (1977)	Isolated from Mastomys natalensis, Mopeia	Spleen	Mouse brain x 4 CDC Vero cell passed x 3 CAMR
M150	"	"	"	"
M152	"	"	"	"
Z478	Johnson et al. (1981)	Isolated Praeomys natalensis, Chiredzi Zimbabwe	Spleen	Isolated in Vero cells CDC passed x 2 CAMR

2.2.2. Mopeia viruses

Five strains of Mopeia viruses were supplied to me by Dr. M. Kiley (Center for Disease Control, Atlanta). Three of these agents were derived from an epidemiological study on rodents carried out in Mopeia, Central Mozambique and two from Zimbabwe (Wulff et al. 1977; Johnson et al. 1981). The five viruses were isolated from pooled liver, spleen and kidney homogenates from different Mastomys natalensis. Original isolations were made in mice after intracerebral inoculation. They were subsequently passed four times I.C. in mice and finally once through Vero cells. The Zimbabwe strain was isolated from Vero cells. It was at this stage that I received the samples.

The viruses were subsequently passed twice in Vero cells and stock preparations made. A summary of passage history is recorded in Table 2. Only pass 3 Vero cell preparations were used in all the studies.

2.3 ANIMALS

2.3.1. Mice

Inbred Balb/c and Porton all purpose mice of outbred stocks of both sexes with an age range between 2 days and 10 weeks were used during these studies. The mice were obtained from Bantim and Kingman Limited, Alderbrough, Yorkshire or from Olac Limited, Bicester, Oxfordshire.

Adult mice were kept in groups of five and suckling mice kept with their mother in individual litters. All mice were housed in polypropylene boxes with stainless steel wire mesh lids. They were allowed a Prepared Mouse Diet (PMD) pelleted (Christopher Hill Group Ltd., Poole, Dorset) and tap water ad libitum. The bedding consisted of sterilized wood chips.

(a) Intracerebral inoculation (I.C.)

Adult and newborn mice (1-21 days old) were lightly anaesthetised in a large jar containing a wad of cotton wool soaked with ether. The solution to be inoculated was taken up in a 0.25 ml syringe with 0.01 ml calibrations fitted with a ½ inch, 27-gauge needle with a short bevel. The mice were secured on a flat surface by holding their head and trunk between thumb and index finger. The skull was swabbed with 1% cetrimide in 70% alcohol. The injection was given by inserting the needle into the cranium at a point half way between the eye and the ear, lateral to the midline. The volume of the inoculum was 0.02 ml.

The procedure used for adult mice was essentially the same as for the newborn mice. With older mice the skull becomes tougher and therefore requires stronger pressure to penetrate. The mice were

therefore put under deeper ether anaesthesia. The volume of the inoculum given was 0.03 ml.

(b) Intraperitoneal inoculation (I.P.)

Mice were injected intraperitoneally with a 25-gauge hypodermic needle in the right hand lower quarter of the abdomen after swabbing the area with 1% cetrimide in 70% alcohol. The usual volume, unless otherwise stated, was 0.1 ml.

(c) Intravenous injection

Intravenous injections were made via one of the lateral tail veins with a 25 gauge hypodermic needle after warming the mice for 2-3 minutes under a chick incubation lamp (150W infra-red lamp, Phillips Electrical Limited, Holland) to dilate the veins.

(d) Collection of blood

Blood samples were taken from the retro-orbital plexes of ether-anaesthetized mice, using a finely drawn-out Pasteur pipette. Gentle suction was maintained with a small rubber teat to encourage blood flow. Unless they were to be exsanguinated, approximately 0.2 ml of blood was taken. The blood was put into small screw capped polypropylene tubes containing heparin (50 units/ml). The sera was separated by centrifuging at approximately 300 g on an MSE Minor centrifuge, removed and stored at -20°C.

(e) Killing of animals

At the end of an experiment or for post-mortem studies, mice were killed by chloroform inhalation.

2.3.2. Guinea Pigs

Dunkin-Hartley strains of guinea pigs weighing between 250-300 grams were used for guinea pig experiments. The pigs were obtained from the Porton animal farm on site.

The guinea pigs were kept in groups of four in metal cages. They were allowed PMD pelleted diet (Christopher Hill Group, Ltd., Poole,

Dorset) and tap water ad libitum. Bedding consisted of sterilised wood chips and hay.

(a) Intraperitoneal injections (I.P.)

Guinea pigs were injected intraperitoneally with a 25 gauge hypodermic needle in the right hand lower quarter of the abdomen after swabbing the area with 70% alcohol. The volume inoculated (unless otherwise stated) was 0.1 ml.

(b) Collection of blood

Guinea pigs were bled under ether anaesthesia by intracardiac puncture. Normally 2 ml was removed unless it was the end of the experiment when as much blood as possible was removed. The blood was either collected into heparin 50 units/ml or allowed to clot at room temperature, the clot then being allowed to contract at $+4^{o}C$ overnight. In both cases the heparinised blood or serum aspirated from the clot was centrifuged at approximately 300 g for 15 minutes in an MSE Minor centrifuge. The serum/plasma, free of erythrocytes, was taken and stored at $-70^{o}C$ for subsequent virus isolation or inactivated at $60^{o}C$/30 mins for storage at $-20^{o}C$ for serology.

(c) Killing of animals

At the termination of an experiment or for post mortem studies, chloroform inhalation was the method used.

2.3.3. Monkeys

The animals used were Rhesus (Macaca mulatta) monkeys. The monkeys were held in individual metal cages, measuring 26" x 40" x 18".

Monkeys were handled only under sedation with intramuscular injection of ketamine hydrochloride (Vetalar, Parke Davis) 60 mg. They were bled from the femoral vein before inoculation with infective material. Monkeys were inoculated intraperitoneally or sub-cutaneously with stock virus at various concentrations (see Results). Rectal

temperatures were recorded every 2 days. Five millilitres of heparinised blood samples were collected aseptically by femoral venepuncture and examined virologically, biochemically and immunologically.

Urine samples were collected aseptically at autopsy by bladder aspiration with syringe and needle which was similarly tested for virus.

Necropsy was carried out on all monkeys shortly after death and various tissues were taken for virological studies and histological examination.

2.4 CELLS

2.4.1. Vero cells

Vero cells obtained from Flow Laboratories Ltd., Irvine,. Scotland, designated African Green Monkey Cells ATCC No. CCL81, were maintained by serial passage at weekly intervals in 75 cm^2 plastic flasks (Nunc). On receipt the level of passage was P133. The growth medium was Medium 199 supplemented with 5% foetal calf serum (FCS) and 25 units/ml of gentamycin. The medium was equilibrated with 5% carbon dioxide in air to maintain a pH of approximately 7.2. Subculture of cells to plastic roller, 25 cm flasks or microtitre plates was established in Plaisners medium (Plaisner et al., 1974) supplemented with 5% F.C.S. and crystamycin. Maintenance medium was Plaisners supplemented with 2% foetal calf serum and 100 units/ml of penicillin plus 100mg/ml streptomycin.

Confluent monolayers were first washed with Phosphate Buffered Saline (PBS) (pH 7.2) and then treated with versene-trypsin solution. After draining the cells were incubated at 37°C until they had detached (approximately 5 minutes). The detached cells were taken up in maintenance media and, after a suitable dilution, were counted in an improved Neubauer haemocytometer. The cell concentration was adjusted to 2 x 10^5 cells/ml in maintenance media. Nunc sterile 25 cm^2 flasks were

seeded with 5 mls, flat bottom 96 well microassay plates (Sterilin) were seeded with 100 µl/well and Lab Tek slides 2 mls/well. The cells were allowed to settle out to form a monolayer at 37°C overnight.

2.4.2. L cells

L cells were obtained from Flow Laboratories Ltd., Irvine, Scotland, Flow designated P569 (mouse areolar adipose tissue). Stocks were maintained and serially passaged weekly in 75 cm^2 plastic flasks in Leibovitz medium (LI5) supplemented with 5% foetal calf serum and crystamycin (Leibovitz, 1963).

Subcultural growth was carried out as outlined under Vero cells.

2.4.3. CV-1 cells

The Rhesus monkey kidney cell lines were obtained from Flow, Irvine Scotland and designated P318. The CV-1 cells are Green Monkey Kidney cell lines originally obtained from Flow and maintained at Porton. The CV-1 cells were treated in exactly the same way as Vero cells (2.4.1.).

2.5 VIRUS ISOLATION

Material to determine antigen levels was cultured in in vivo and in vitro host systems.

2.5.1. Preparation of Inocula

2.5.1.1. Urine specimens

The pH of the urine was adjusted to 7.0-7.4 with $NaHCO_3$ if necessary. Urine was concentrated by centrifuging at 2,500 r.p.m. for 20 minutes, and the sediment resuspended in one-tenth of the original volume of supernatant urine. A portion of this concentrate was treated with penicillin (10,000 units) and streptomycin (50,000 µg/ml). A portion of the concentrate was mixed with an equal volume of 70% sorbitol and stored at -70°C. The sample was diluted in Plaisners medium supplemented with 2% heat treated FCS and inoculated into 25 cm^2 flasks and microassay plates.

2.5.1.2. Tissue suspensions

Tissues taken at autopsy were homogenised into a suspension for inoculation into cell cultures or in mice.

A representative sample of the tissue was weighed and transferred to a mortar and ground into a suspension with Plaisners medium containing 1% FCS. The suspension was then centrifuged at 2,500 r.p.m. for 10 minutes at $+4^{o}C$. The supernatant fluid was removed and treated with antibiotics, i.e. to each millilitre of clarified tissue suspension is added 0.1 ml of a solution containing 10,000 units penicillin/ml and 50,000 µg streptomycin/ml. The samples were stored at $-70^{o}C$.

Samples from cell culture were titrated by 10 fold amounts in 25 cm^2 flasks and microassay plates and incubated at $37^{o}C$. A 0.1 ml volume was inoculated I.C. into adult mice or I.P. into guinea pigs.

2.5.1.3. Serum

Serum collected as in Section 2.3 was diluted 10-fold in 2% Plaisners medium and 0.1 ml amounts inoculated into 25 cm^2 flasks and/or microassay plates.

2.5.2. Titration of Virus in Monolayer Cell Culture ($TCID_{50}$) Endpoints

Determination of $TCID_{50}$ (tissue culture infectious dose) endpoints in all virus suspensions were carried out using logarithmic dilutions prepared in maintenance medium. Each dilution was added in volumes of 0.5 ml/25 cm^2 flask on 100 µl/well of a microassay plate. Each dilution placed in flasks was completed in duplicate and microassay plates in quadruplicate. The inoculated cultures were incubated at $37^{o}C$ and observed daily over a 7 day period for specific viral cytopathic effect. Confirmation of endpoint was made using immunofluorescence. The $TCID_{50}$ endpoint was calculated by the method of Reed and Muench (1938).

2.5.3. Titration of virus after chemical inactivation

After treatment, the level of infective virus was estimated by

a modification of the virucide floating technique (Fig. 14, van der Groen et al., 1980). A 2% agarose block (1 cm^2) was mounted on the edge of a glass slide. A known quantity of virus suspension was put on the surface of the agarose, and allowed to diffuse and evaporate until the surface was completely dry (approximately 10 minutes). Two drops of 0.5% (w/v) formvar (BDH) dissolved in 1,2-dichloroethane (Merck) were addded and left for a few seconds. Excess formvar was then removed with blotting paper. When the film was dry the edges of the block were cut with a scalpel. By placing the block into the inactivant solution a film containing the virus was released: this moment of virus-inactivant contact was noted. After a known time the film was removed with a sealed glass Pasteur pipette.

Excess inactivant still present was removed by gently bonding the film against the side wall of a tube. The film was transferred into 1 ml tissue culture medium, disrupted with a sealed Pasteur pipette and sonicated to release residual virus into suspension. The suspension was then inoculated into tissue culture in order to determine residual infectious virus.

Control experiments were included where the inactivant was replaced by tissue culture medium.

2.6. SEROLOGICAL AND IMMUNOCHEMICAL PROCEDURES

2.6.1. Serum neutralization assay

Serum obtained from both animals and human sources were tested for neutralization antibodies by the microneutralization test.

Tests were carried out in sterile microtitre plates (Sterilin Ltd., Teddington, Middlesex). Serum and virus dilutions were prepared in Plaisners medium, containing 2% FCS (heat treated at 56°C for 30 minutes). Immune and pre-immune sera for test were treated at 56°C for 30 minutes. Neutralization tests were performed by mixing 0.5 ml of a 1/5

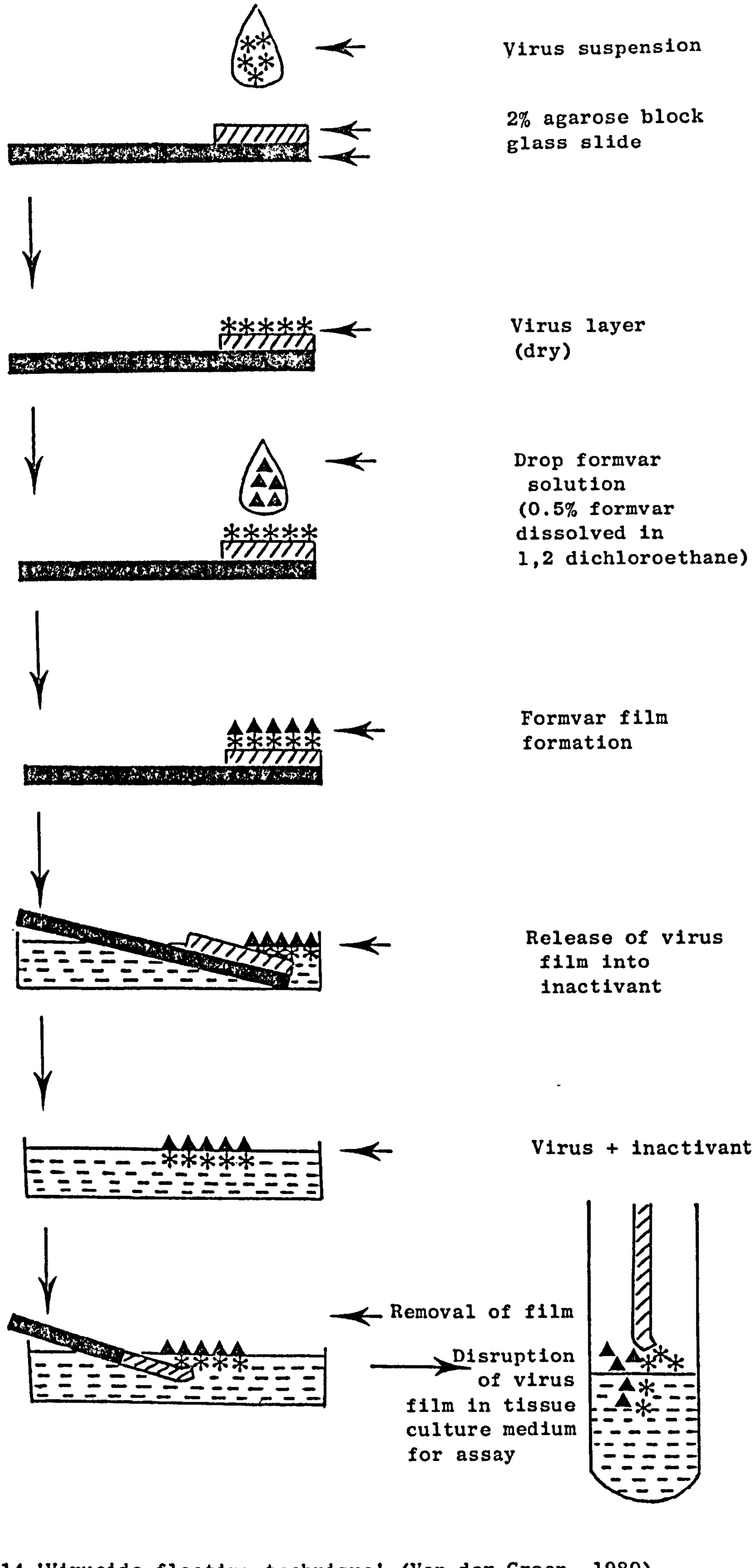


Fig. 14. 'Virucide floating technique' (Van der Groen, 1980)

serum dilution (containing 1/10 guinea pig complement C') with 0.5 ml of two 10-fold serial dilutions of virus. After incubation at 37°C for 60 minutes 100 µl volumes of the mixture was distributed into 10 wells of a micro-assay plate containing a monolayer of Vero cells. After 1 hr incubation at 37°C 100 µl of media was added. The plates were incubated at 37°C and examined daily for development of cytopathic effect (CPE). Final readings were made on the 10 day following incubation, and the 50% end-point calculated by the Reed-Muench method.

Neutralizing antibody was expressed as a $\log_{10}$ neutralizing index (LNI) which represents the difference between the titre of the virus in the presence of normal control serum and the titre of the virus in the presence of the test (immune) serum.

$$LNI = \log_{10} (TCID_{50} \text{ in control}) - (TCID_{50} \text{ in test serum})$$

2.6.2. Immunofluorescent test

The indirect immunofluorescent test was to measure antibody levels in studies involving mice, guinea pigs, monkey and human serum. The technique was also used to identify isolations of viruses from body fluids and tissues.

2.6.2.1. Antigen slides for Antibody determination

Antigen-containing cell preparations were prepared on Teflon-coated slides with 15 circular areas or spots (Flow, Irvine, Scotland). L cell cultures under fluid medium were infected with any of the required viruses under study at a multiplicity of infection (M.O.I.) of 0.1. Three or four days later, regardless of the presence or absence of CPE, the infected cultures were harvested; the cells were dispersed with 0.25% Trypsin/Versene, washed x 3 with PBS (pH 7.2) and resuspended in a volume of 0.01% Bovine Serum Albumin (BSA)/PBS, giving a final concentration of approximately 10^6 cells/ml. The suspension was mixed and a drop placed on each spot of the slide (10^3 cells approximately). The slides were held at

room temperature for 15 minutes until dry, then fixed in acetone at +4°C for 10 minutes, dried in air, stored in plastic slide boxes and screw capped tins at -70°C until used.

2.6.2.2. Antigen detection

Slides, microassay plates and flasks used for isolation and confirmatory IFA testing were prepared by the following technique. Cell monolayers under fluid media were grown on chambers slides (Lab Tek) plates or flasks. Each chamber of the Lab Tek slides was seeded with 2 x 10^5 cells in a volume of 0.8 ml growth medium. Flasks and microtitre plates were prepared as described in Section 2.4.1. After 24 hours they were infected with tissue homogenates or sera from experimental animals with a range of dilutions (10^{-1} - 10^{-8}). Between 4-7 days the chambers were removed, slides thoroughly rinsed with PBS, fixed in Acetone at +4°C for 10 mins. dried in air and stained or stored at -70°C. The microassay plates were fixed with pre-coated methanol and tested immediately by the IFA. Cells from flasks were prepared as outlined in 2.6.2.1.

2.6.2.3. IFA procedures

The serum for antibody determination was diluted in PBS pH 7.2 and overlayed on infected cells or Lab Tek slide chambers (see Section 2.1) for 30-40 mins. at room temperature. Slides were then washed x 3 in changes of PBS for a total of 5 minutes. The material was reacted with an optimal dilution of fluorescein isothiocyanate conjugate prepared against the species of immunoglobulin in which the viral antisera was prepared. The conjugates used were obtained from Miles Research Products (Slough, England). The reaction times were 30-40 minutes. Slides were washed three times in PBS (10 minutes), twice in distilled water (3 minutes), dried and read immediately.

Microassay plates and slide chambers received the same treatment with one exception. After the final wash in PBS, one drop of 50% glycerol in PBS was added to each well and a coverslip applied.

The fluorescence was observed using a Reichert A O fluorostar microscope (Buffalo, New York 14215) at a magnification of x 160 for screening and x 400 for end-point determination with incident illumination provided by a 50W mercury vapour lamp. The filtration system utilized a fluorocluster 2072 which contained a BG12 + KV418 exciter, OG 515 barrier and dichroic beam splitter reflecting all the desirable short wave excited light down through the objective to the specimen (500 mm).

2.6.3. Immunochemical studies on virus structural proteins using the "Western blot" technique

2.6.3.1. Viral growth and purification

Confluent monolayers of cells in 1750 cm^2 plastic roller bottles (Falcon, Beckton-Dickinson, Ltd.) were infected at a multiplicity of 0.1 in a volume of 10 ml of Plaisners Modified Eagles Medium supplemented with 2% FCS and antibiotics. After 60 minutes adsorption 150 ml maintenance medium was added and incubation continued at 37°C. Medium was harvested and replaced on day 2 post-infection and finally harvested on day 3. Virus was purified by a method similar to that of Kiley et al., (1981). The pooled medium was centrifuged at 12,000 x g for 10 minutes at 4°C to remove cells and debris and the supernatant brought to 5% NaCl and 7% polyethylene glycol 6000 (BDH, Ltd.) by addition of solids with gentle stirring at 4°C. The pellets were then resuspended in 1/100th the original volume of 200 mM glycine, 100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5 (GTNE) and clarified by centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatant was layered on 30 ml glycerol-tartrate gradients (Obijeski et al., 1974) in heat-sealable tubes for the Beckman 60 Ti rotor and centrifuged at 44,000 x g for 16-18 hours at 4°C using the slow acceleration and deceleration facilities of a Sorvall OTD 65 ultracentrifuge. The visible virus band about half way down the tube was recovered, diluted in GTNE and pelleted by centrifugation at 27,000 x g for 90 minutes at +4°C. The pellets were resuspended in a small volume of GTNE, and stored at -70°C.

2.6.3.2. Infected cell lysates

Confluent monolayers of Vero or CV-1 cells were infected at a multiplicity of 0.1 with virus diluted in maintenance medium. For direct lysis in SDS the cells were dissolved in electrophoresis sample buffer (ESB) (1% SDS, 1% 2-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue, 50 mM Tris-HCl, pH 6.8) and heated at 60°C for 2 hrs to denature the proteins and inactivate the virus. For lysis under denaturing conditions the cell monolayer was exposed to PBS containing 1% Triton X-100 and 1% SDS for 5 minutes at room temperature.

2.6.3.3. Polyacrylamide gel electrophoresis

Samples were treated with 1% SDS, 1% 2-mercaptoethanol, 100 mM Tris-HCl, pH 6.8, 15% glycerol, 0.01% bromophenol blue and heated at 100°C for 2 minutes prior to analysing or 8-15% polyacrylamide concave exponential gradient gels using the buffer system of Laemmli (1970). The gels were run at 75 mA for 1.5 mm thick gels or 40 mA for 0.75 mm gels. The stain Kenacid blue R (BDH, Ltd.) in 25% isopropanol, 10% acetic acid was used for protein identification. Restaining was achieved with 10% isopropanol and 10% acetic acid. Molecular weights were estimated by comparison of electrophoretic mobilities with those mixtures of standard protein markers, either unlabelled (Sigma) or ^{14}C-methylated (Amersham International).

The proteins separated on gels were transferred to nitrocellulose sheets (BA85, Schleicher and Schull) as described by Towbin et al. (1979) except the transfer buffer was 96 mM glycine, 12.5 mM Tris, 20% isopropanol and current was passed at 200-300 mA for 14-16 hours. Monitoring of the transfer to nitrocellulose sheets was made by staining with Amido black (Towbin et al., 1979) and destaining in 10% acetic acid, 10% isopropanol.

2.6.3.4. Glycoprotein detection

Nitrocellulose transfers were incubated for 1 hour in PBS, 0.5% Triton X-100 containing 50µM Mg^{2+}, Mn^{2+} and Ca^{2+} ions (GS buffer) and 10µg concanavalin A (Pharmacia) per ml with constant agitation. The transfers were washed 5 times in GS buffer and incubated in GS containing horseradish peroxidase (Sigma 50 µg/ml) for 1 hour before repeating the washing cycle. The positions of the glycoprotein bands were revealed by incubation with aminoethlycarbazole (200 µg/ml), H_2O_2 (0.03%) in 50 mM sodium acetate buffer pH 5.0 (Clegg, 1982).

2.6.3.5. Immunological detection of proteins using nitrocellulose transfers

Nitrocellulose transfers were incubated at room temperature with constant agitation in PBS containing 2.5% BSA, 5% FCS 1% Triton X-100 and 0.1% SDS (AB buffer) in which antisera or monoclonal antibodies had been suitably diluted. After 2.4 hours the transfers were washed 3 times in PBS containing 0.05% Triton X-100 and incubated in AB buffer containing a 1/1000 dilution of horseradish peroxidase conjugated anti-globulin of the appropriate species (Miles) for 1 hour and washed. Antibody-binding bands were revealed by incubation with aminoethylcarbazole as for the glycoprotein detection method.

2.7 ELECTRON MICROSCOPY

2.7.1. Negative staining

(a) Infective Blood

Blood appeared to be one of the richest sources of virus in body fluids, with titres of approximately $10^{6.5}$ $TCID_{50}$/ml at the height of viraemia with Lassa virus (Buckley and Casals, 1970). Serum obtained from guinea pigs, mice and monkeys was mixed with 3% gluteraldehyde in cacodylate buffer and held at room temperature for 24 hours. The sample encased in Beckman sealed tubes, was pelleted at 18,000g for 2 hours in a Beckman L8 refrigerated centrifuge using a 40 fixed angle rotor. A small

amount of distilled water was used to resuspend the pellet. A drop of the specimen was placed on to copper specimen grids coated with Formvar. The excess was removed with filter paper after 2-3 minutes. Drops of P.T.A. (pH 6.4-7.2) were placed on the grids, the excess being removed with filter paper. The grids were then stabilised with a film of evaporated carbon and examined in a Phillips EM 400T electron microscope.

(b) Tissue Culture Supernatant

Monolayers of cells in Nunc 25 cm^2 flasks were infected with Lassa and Mopeia viruses. At various points during the infection, cell culture fluids were mixed with an equal volume of 3% glutaraldehyde/cacodylate buffer at room temperature for 24 hours and then treated as for plasma (Section 2.7.1.(a).

2.7.2. Tissue for Electron microscopy

During pathogenesis studies with guinea pigs, mice and monkeys infected with Lassa and Mopeia viruses, tissue was removed at post-mortem for electron microscopy. The tissues of main interest were: liver, spleen, kidney, lymph nodes, pancreas, heart, lungs, small and large intestine.

2.3 mm cubes were prepared from the organs and placed in 3% glutaraldehyde in cacodylate buffer in sealed containers. Forty-eight hours later, 1 mm cubes were cut from these and placed in fresh phosphate buffered glutaraldehyde. These were later washed in repeated changes of sucrose/cacodylate buffer (pH 7.4) for 18 hours. The washed specimens were then post fixed in 1% osmium tetroxide in cacodylate buffer and distilled water for 2 hours, then washed for a similar period of time in distilled water. Dehydration was carried out in graded methanol, specimens then spending 30 minutes in 3% uranyl acetate in 30% methanol. Final embedding was carried out in Durcupan (EM Scope), via graded propylene oxide mixtures. After 48 hours polymerization at 60°C, sections

were cut on a Reichert (OMU 2) or a Cambridge Huxley Ultramicrotome, and stained with lead nitrate before viewing with a Phillips EM 400T at CAMR or a JEOL 100 CX at the London School of Hygiene and Tropical Medicine.

2.7.3. Tissue cells for E.M.

To study developmental growth kinetics, cells were infected with a multiplicity of infection (MOI) of 0.1 of Lassa and Mopeia viruses.

Infected cells were mixed with 5 ml of 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and held for 24 hours. During this time both cells and container were exposed to formalin vapour before being taken from the Maximum Containment Laboratory.

The cell pellet produced by the first centrifugation at 500g for 15 minutes was washed overnight in 0.1M cacodylate/0.2M sucrose buffer, pH 7.4. After several washings the cells were spun at 500g for 5 minutes to produce a pellet, which was post fixed in 1% osmium tetroxide for 2 hours. The cells were then washed in buffer and pelleted in 2% agar (Oxoid No. 1) and further processed as outlined in 2.7.1(a).

2.8. HISTOLOGICAL EXAMINATION

Mice and guinea pigs were killed with ether, monkeys with intravenous nembutal. Whether natural or induced death, organs were removed immediately, and portions of the following tissue were removed:- myocardium, duodenum, ileum, caecum, colon, pancreas, salivary glands, lung, liver, spleen, kidney, adrenal glands, testes, and lymph nodes. Since Lassa is so readily transmitted to man via skin wounds with very serious consequences, the brain and spinal cord were not removed because of the risk of piercing a glove on spikules.

Tissues were fixed in 10% buffered neutral formalin and embedded in paraffin wax. Sections were cut at 5 μ and stained by haematoxylin and eosin (H and E).

2.9 REAGENT PREPARATION

2.9.1. Immune serum and ascitic fluids

2.9.1.1. Mice.

Arenavirus-infected newborn mouse brain tissue suspensions were freshly prepared in physiological saline (pH 7.2) to a final concentration of 10%. Alternatively, tissue culture derived virus was used. On day 0, 0.3 ml of virus (10^6 $TCID_{50}$/ml or 10^5 LD_{50}/ml) was inoculated i.p. into 4-5 mice. On day 20-25, a second similar injection of fully active virus was given and a test bleed was carried out 7 days later.

A third injection of virus similar to the second was given at this point followed 2 days later by an i.p. injection of 0.1-0.2 ml of ascites from a mouse bearing sarcoma 180 cells. Sarcoma fluid was removed by parencentesis 7-9 days after the administration of sarcoma 180 cells. The ascites were pooled and allowed to clot at room temperature, centrifuged at 3200 x g for 30 minutes. The clear fluid was collected and stored at -20°C until used.

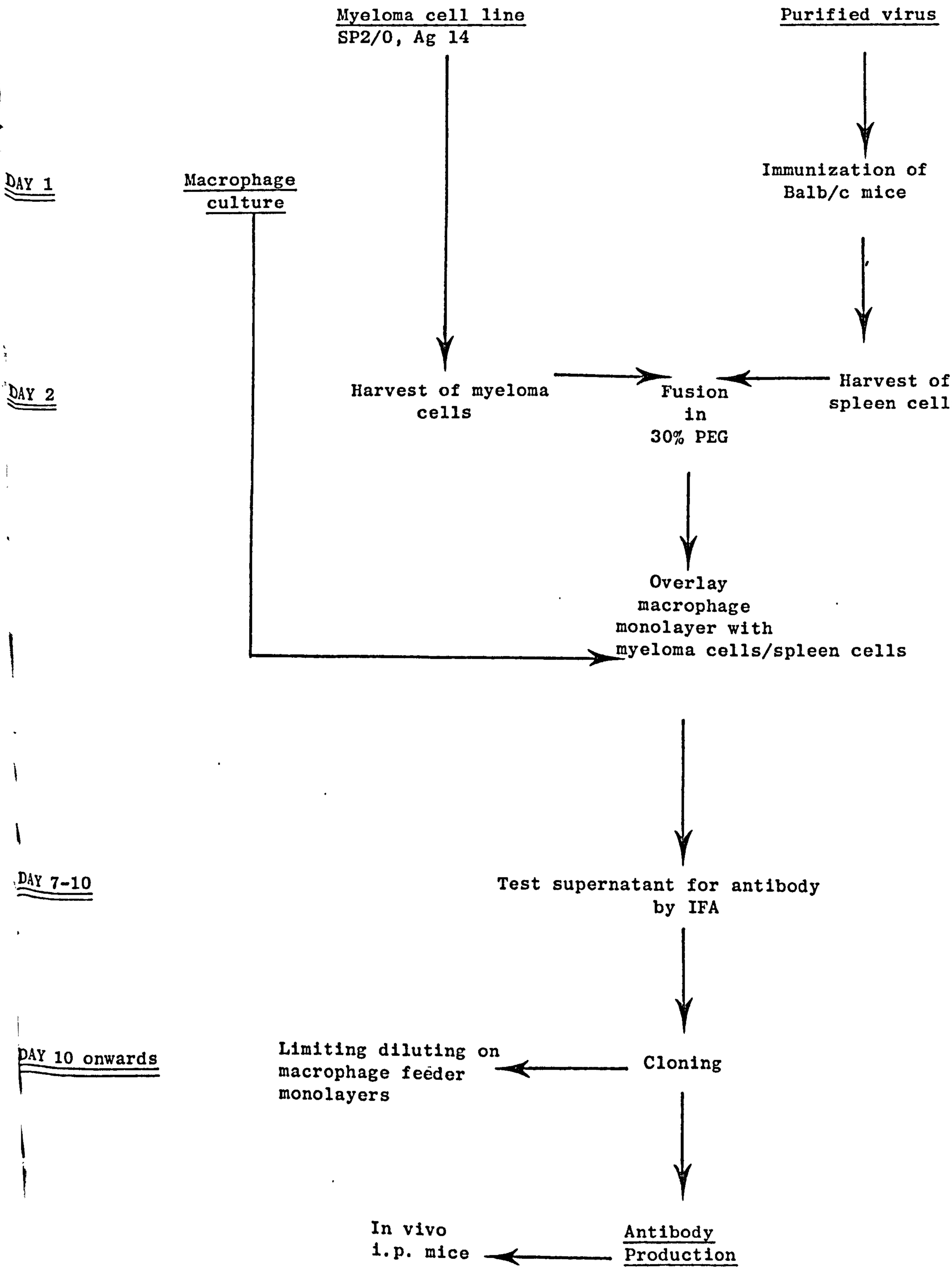
2.9.1.2. Guinea pigs.

An LGA 391 inoculum of 10^5 $\log_{10}$ virus-infective tissue culture results in a 75% mortality; the survivors were bled 28-30 days after infection yielding serum with a workable CF and IF antibody. The serum obtained after 60 days contained neutralizing antibodies which were used as a standard reagent. No advantage was gained by repeated inoculation. Serum was placed in 0.2 ml aliquots, serologically standarized and stored at -70°C or lyophilized and stored at -20°C until used.

2.9.2. Monoclonal antibodies.

The technique for preparing monoclonal antibodies by fusion of spleen cells derived from immunized mice and murine myeloma cells was based on the work of Kohler and Milstein (1975). Hybridoma cell lines were generated by the fusion of spleen cells from immunised Balb/c mice with

Fig. 15 Basic Steps in the Production of Monoclonal Antibodies



SP2 mouse myeloma cells (Schulman et al., 1978) in Roswell Park Memorial Institute (RPMI) 1640 (Moore 1967) containing hypoxanthine-aminopterin-thymidine (HAT) medium (Littlefield 1964). An outline of the basic steps in the production of monoclonal antibodies is shown in Fig. 15.

The protocol for immunization involved priming Balb/c mice i.p. with 0.1 ml tissue culture supernate containing 6 $\log_{10}$ $TCID_{50}$ of virus. Four weeks later, 0.1 ml booster doses of antigen (6 $\log_{10}$ $TCID_{50}$) were administered over three consecutive days, i.e. Day 1 i.p., Day 2 i.p., day 3 i.p. and i.v. Twenty four hours after the final boost of antigen, the animals were killed and the spleens removed aseptically. The spleen cells were manually separated, washed, counted and adjusted to 10^8/ml in RPMI 1640 without any serum.

Peritoneal macrophages were obtained from adult mice by injecting approximately 6 ml of an 0.34 M-sucrose solution peritoneally and, after gentle massaging of the abdomen, withdrawing the fluid. The sucrose-containing macrophages were collected in plastic universals and after centrifugation the cell concentration was adjusted to 5×10^5 cells/ml in RPMI 1640 + HAT.

The SP2/0 AG14 myeloma cells were grown in stationary cultures (75 cm^2 flasks) in RPMI 1640 media containing 20 mM Hepes, 15% FCS and glutamine, usually allowing a cell density of around 10^5/ml. In preparation for fusion, the cells are harvested and washed twice in RPMI 1640 without FCS and adjusted to 10^7 cells/ml.

For fusion, the technique described by Frazekus de St. Groth and Scheidegger (1980) was used as a guide. In general, spleen and myeloma cells were mixed together in serum free RPMI 1640 at a ratio of 10 : 1 (spleen : myeloma). The combined cell suspension was centrifuged (400 x g for 5-10 min) and supernatent removed. Fusion was initiated by slowly adding 1 ml 50% polyethylene glycol (PEG) 4000 (Serva) in saline, gently

stirring over 1 minute and transferring the centrifuge tube to a 37°C water bath for 90 seconds (Galfre and Milstein, 1981). The solution was gradually diluted to 10 ml, by gently stirring over several minutes with serum free RPMI 1640. The cells were then pelleted (200 g, 10 minutes) washed and resuspended in 12 ml RPMI 1640 medium containing HAT medium (0.1 mM-hypoxanthine (H), 0.4 µM aminopterin (A) and 16 µM thymidine (T), 20% heat inactivated foetal calf serum and antibiotics). Fifty µl amounts were then distributed into the wells of a 96-well plastic culture tray (Linbro), which had previously been seeded with 50 µl HAT medium containing peritoneal macrophages at a concentration of 5×10^5 cells/ml. The cultures were then inspected 7 to 10 days later and those wells which showed growth of cells were fed with fresh HAT medium, in some cases by removal and replacement of medium. At this stage, the culture supernates were screened for the secretion of antibodies by IFA. Those cultures showing positives were cloned by limiting dilution in further 96-well plates previously seeded with peritoneal macrophages. This process was repeated twice. The cells derived from wells showing growth of single colonies were transferred to 24-well plastic trays (Linbro) and then 6-well trays (Linbro) and were again screened by IFA. Those clones which still retained their ability to produce specific antibodies were further amplified before storing in liquid nitrogen or producing mouse ascites. Ascitic fluids were made in Balb/c mice by inoculating 10^6 cells i.p., and withdrawing fluid and/or tumour 10-15 days later.

Monoclonal antibodies to LCM were kindly donated to me by Dr M Buchmeier at the Scripps Clinic and Research Foundation. Monoclonals derived from Pichinde, Junin and Tacaribe were obtained from Dr C R Howard and Dr A C Chanas, London School of Hygiene and Tropical Medicine.

2.9.3. Human Sera

All sera used were derived from patients with proven Lassa infection.

2.9.4. Complement-fixing antigens

Brains from 2-5 day old suckling mice inoculated i.c. with Lassa or Mopeia viruses, were harvested and pooled. The brains were homogenized in chilled 8.5% sucrose in IM Tris (hydroxymethyl) methylamine buffer pH 9 containing 1% β-propiolactone (BPL) puriss grade (Fluka AG, Glossop, Derbyshire) using a glass homogenator, followed by sonication using a soni-probe (Dawe Instruments Ltd., London). This was kept at +4°C for 18 hours. The sucrose buffer was removed and added dropwise with mechanical stirring to 20 volumes of chilled acetone (analytical grade). The mixture was centrifuged at 500 x g for 5 minutes. The supernatant fluid was removed and discarded. A volume of acetone equal to that first used was added to the sediment, and centrifuged at 500 x g for 5 minutes, the sediment subsequently being dried under oil-pump vacuum. After 3-4 hours 0.9% NaCl equal to 40% of the volume or the original homogenate was added to the dry sediment. This solution was held at +4°C overnight, centrifuged for 1 hour at 12,800 x g. The supernatant fluid constituted the antigen and was stored in 0.5 ml or 1.0 ml amounts at -20°C.

Alternatively an infected cell culture CF antigen was made. Roller bottles or 75 cm^2 tissue culture flasks were infected with 0.1 ml MOI, the cells and fluid being harvested between 6-8 days. One volume of IM Tris buffer pH 9 containing 1% beta-propiolactone was added to 9 volumes of culture fluid. After 18 hours at +4°C the material was centrifuged at 500 x g for 15 minutes to sediment the cellular debris. The supernatant fluid constitutes the antigen.

2.10 CLINICAL BIOCHEMISTRY

Evaluation of changes in animal serum components were determined using test kits (Boehringer Ingelheim Ltd., Southern Industrial Estate,

Bracknell, Berkshire). The activities of enzyme and substrates in sera studied are listed below:

alkaline phosphatase (AP)

glutamine oxaloacetate transaminase (GOT)

glutamine pyruvate transaminase (GPT)

lactate dehydrogenase (LDH)

α-hydroxybutyrate dehydrogenase (α-HBDH)

creatine kinase (CK)

γ-glutamyl transpeptidase (γ-GT)

creatinine

urea

total protein

triglycerides

cholesterol

All the above analyses were carried out in an LKB Kinetic Analysis System (Ultralab) according to the LKB method guide (LKB Booklet 1975).

2.11. PHYSICOCHEMICAL STUDIES

2.11.1. Thermal stability

Stock Lassa and Mopeia viruses were added to human sera, guinea pig sera or phosphate buffered saline containing different percentages of foetal calf serum. A water bath in which the water is circulated and the temperature controlled to within 1°C was used. A sterile glass tube of 10 ml capacity was placed in the water bath set at a selected temperature. When the temperature of 5 mls of support medium + virus reached that of the water bath, it was considered zero time and 0.5 ml samples for virus assay were removed at predetermined time intervals and placed in an ice-water bath until they were used.

2.11.2. Ultra violet light inactivation

Stock tissue culture derived Lassa and Mopeia viruses were diluted in phosphate buffered saline pH 7.3 which contained varying levels of human

or guinea pig sera. The final concentration of viruses was adjusted to 10^6 $TCID_{50}$/ml for easy comparison.

Each solution was dispensed into plastic petri dishes having a diameter of 50 mm. The depth of this fluid was approximately 3-3.5 mm. The dishes were placed on a rocking shelf with a rocking cycle of 20 complete cycles per minute, and at a distance of 15 mm from a 15 watt Phillips germicidal tube giving an intensity of 600 microwatts/cm^3. Exposure times ranged from 0 to 10 minutes, samples being removed and assayed for virus at intervals.

2.11.3. pH sensitivity

Infectious tissue culture fluid was adjusted to the desired pH level by the addition of 1 molar HCl or 1 molar NaOH. To facilitate rapid manipulation, the amounts of these reagents required to adjust a given volume of infectious tissue culture fluid were determined in advance. In the inactivation experiments the predetermined amounts of the reagents were added to the measured volume of infectious tissue culture fluid under gentle agitation whereupon the final pH was determined and the temperature noted. Samples for virus assay were removed at predetermined time intervals and re-adjusted to pH 7.3. Adjustment and re-adjustment of the pH required approximately 1½-2 minutes.

2.11.4. Sensitivity to solvents

The effects of solvents on viral infectivity were tested at 37°C by adding equal amounts to stock infectious tissue culture fluid. The samples were continually mixed at +37°C by a magnetic stirrer for 1 hour and titrated in tissue culture. A list of solvents and their source is shown in Table 3.

TABLE 3. LIST OF SOLVENTS USED

Solvents	Final concentration	Source
Ether	50%	BDH Poole, Dorset
Chloroform	10%	"
Sodium desoxycholate	0.5%	"
Acetone	50%	"
Alcohol	50%	J Burrough Ltd., London

2.11.5. Glutaraldehyde and Formaldehyde inactivation

Glutaraldehyde 25% solution in water (Taab, 52 Kidmore End Road, Emmer Green, Reading) and formaldehyde 40% w/v (Fisons Scientific, Loughborough, Leics.) were used as stock solutions.

The effect of glutaraldehyde and formaldehyde on Lassa and Mopeia was studied by following the rate of inactivation at pH 7.4 at temperatures of $4^{o}C$, $25^{o}C$, and $37^{o}C$. For each temperature combination, a series of experiments using different concentrations of formaldehyde and glutaraldehyde in the range 0.2% - 10% was used. The control experiments excluded the use of formaldehyde or glutaraldehyde. Each concentration employed a mixture using tap water or 10% foetal calf serum in Plaisner's medium as a diluent for the stock suspension of virus. Residual amounts of infective virus were measured at intervals after the initial exposure. Their treatment and titration followed the procedure found in 2.11.4.

2.11.6. βpropiolactone inactivation (BPL)

An aqueous solution of BPL (Fluka AG, Glossop, Derbyshire) (puriss grade), usually 10% v/v, was first made with distilled water at $4^{o}C$, the solution being kept in an ice bath to reduce the rate of BPL hydrolysis. The inactivation of LGA 391 and Mopeia viruses with various final concentrations of BPL (0.025 - 0.3%) was determined at $4^{o}C$, $22^{o}C$ and $37^{o}C$.

The effect of BPL with various combinations of concentrations and temperatures was determined in blood, sera, mouse brain and tissue culture fluid.

The rate of BPL hydrolysis was based on the absorbance of BPL in the infra-red spectrum, which showed a characteristic peak of 5.5μ in a solution of carbon tetrachloride (CCl_4). The size of this peak was used to measure the amounts of BPL extracted by CCl_4. Hydracrylic acid, the hydrolysis product of BPL in an aqueous solution, showed no peak at this wavelength. A 1 ml sample of test material was added to 2g of anhydrous sodium sulphate (Na_2SO_4) and 3 ml CCl_4, in a mortar, previously cooled to 4^oC. The crystalline (hydrated sodium salt) product of the mixture was ground with a pestle. The solution was coarse-filtered through a sintered glass funnel, gentle suction being applied to pull through the maximum amounts of CCl_4 containing the BPL. The mortar was washed three more times with 2 ml CCl_4, each washing being poured over the Na_2SO_4 and through the filter. The final filtrate was made up to 10 ml with CCl_4 and mixed. Samples of CCl_4 extract were placed in an infra-red spectrophotometer (Hilger-Watts 'Infragraph' H1200) and peaks at 5.5μ were measured.

In this study the amount of BPL remaining during the course of hydrolysis at different temperatures (4^oC, 22^oC and 37^oC) in various solutions (phosphate buffered saline, tissue culture fluid, serum or blood) was measured with the method described.

2.11.7. Nucleic-Acid Determinations

5-bromodeoxyuridine (BUDR), obtained from the California Corporation for Biochemical Research, was stored frozen (-20^oC) as a 10^{-8} molar (M) solution in distilled H_20. The BUDR solution was diluted in Plaisners tissue culture fluid to give varying dilutions 10^{-6} - 10^{-2} M. Plaisners medium supplemented with 2% inactivated foetal calf serum

0.02M glutamine and BUDR was placed on the cells previously adsorbed with virus. Samples of fluid were periodically sampled for viral infectivity.

LGA 391, Mopeia and Vaccinia viruses were titrated in Vero cells in the presence of Plaisners medium containing 100 µg of 5-BUDR and samples taken over 6 days.

2.11.8. Effect of Actinomycin D

Vero cells were infected with virus at a multiplicity of 0.1. After 2 hours adsorption at 37°C the monolayers were washed and treated with doses of actinomycin D ranging from 0.01 - 0.5 µg/ml. After 60 hours' incubation, yields of extracellular virus were assayed by titration in microassay plates. A second set of culture was treated in the same way by actinomycin D added simultaneously with the virus.

2.12. Sources and make-up of reagents used

2.12.1. MEM (Eagle) - Plaisner Modified (Plaisner et al. 1974)

	mg/litre
L-arginine HCl	126.4
L-cystine	24.02
L-Histidine $HClH_20$	41.9
L-isoleucine	52.5
L-leucine	52.5
L-lysine HCl	73.06
L-methionine	14.9
L-phenylalanine	33.02
L-tyreonine	47.64
L-tryptophan	10.2
L-tyrosine	36.22
L-valine	46.9
D-Ga pantothanate	1.0
Choline bitartrate	1.8
Folic acid	1.0
i-inositol	2.0
Nicotinamide	1.0
Pyriboxal HCl	1.0
Riboflavin	0.1
Thiamin HCl	1.0
$CaCl_2$ $2H_20$	200.0
Galactose	800.0
Sodium pyruvate	200.0
$MgS0_4$ $7H_20$	98.0
KCl	400.0
NaCl	6800.0
$NaH_2PO\text{-}H_20$	140.0
Phenol Red	10.0
Sodium succinate	1000.0
Succinic acid	750.0

pH 7.4 with 6% NaOH

Sterilize by filtration

Replace used medium with Plaisners after 5 days' incubation

2.12.2. L-15 (Gibco Biocult Cat. No. 430-1300) (Leibovitz, 1963)

Components	Mg/litre
Inorganic salts	
$CaCl_2$(anhyd)	140.000
KCl	400.000
KH_2PO_4	60.000
$MgCl_2$ (anhyd)	93.800
$MgSO_4$ (anhyd)	97.670
NaCl	8000.000
Na_2HPO_4 (anhyd)	190.123
Other components	
D(+) Galactose	900.000
Phenol Red	10.000
Sodium pyruvate	550.000
Amino acids	
DL Alanine	450.000
L-Arginine (free base)	500.000
L-Asparagine	250.000
L-Cysteine (free base)	120.000
L-Glutamine	300.000
Glycine	200.000
L-Histidine (free base)	250.000
DL-isoleucine	250.000
L-Leucine	125.000
L-Lysine (free base)	75.000
DL-Methionine	150.000
DL-Phenylalanine	250.000
L-Serine	200.000
DL-Threonine	600.000
L-Tryptophan	20.000
L-Tyrosine	300.000
DL-Valine	200.000

Vitamins

DL-Ca pantophenate	1.000
Choline chloride	1.000
Folic acid	1.000
Inositol	2.000
Nicotinamide	1.000
Pyridoxine HCl	1.000
Riboflavin-5'-phosphate sodium	0.100
Thiamine monophosphate	1.000

2.12.3. Foetal calf serum

Virus and mycoplasma screened in 500 ml amounts obtained from:

Gibco Biocult Ltd, 3 Washington Road,

Paisley, PA3 4EP, Scotland.

2.12.4. Phosphate buffered saline (Dulbecco A) PBS

Na Cl	8.0g
K Cl	0.20g
$Na HPO_4$	1.15g
$KH_2 PO_4$	0.20g
Phenol red (2% solution)	0.10g
H_2O	to 1000 ml

pH 7.4

Sterilized by autoclaving

2.12.5 Versene-trypsin solution (0.05% versene, 0.2% trypsin)

* Versene (5% solution)	1.0 ml
** Trypsin (2% solution)	10.0 ml
$NaHCO_3$ (5.5% solution)	2.5 ml
PBS	86.5 ml

pH 7.6

Sterilized solutions were mixed aseptically.

* Versene (5% solution)

Ethylenediaminetetraacetic acid (disodium salt) 50g

H_2O to 1000 ml

Sterilize by filtration

** Trypsin (2% solution)

Trypsin 1:250 (Difco, Michigan, USA) 20g

PBS to 1000 ml

Sterilize by filtration

2.12.6 Crystomycin (Gibco)

Penicillin - Streptomycin

Lyophilized. Prepared in normal saline

Pencillin (Base) 5,000 units/ml

Streptomycin (Base) 5,000 mcg/ml

(Gibco Biocult Ltd., 3 Washington Road,
Paisley PA3 4EP, Scotland)

2.12.7. Neutral formol saline for histopathology

10% solution:-

* 22.75 gm Sodium dihydrogen phosphate (NaH_2PO_4)
(anhydrous)

** Formaldehyde 40% 500 ml

Dist. H_20 4,500 ml

* BDH Chemicals Ltd, Poole

** Formaldehyde solution 37/40% w/v

Fisons Scientific Apparatus, Loughborough, Leics.

2.12.8. 3% Glutaraldehyde for E.M. fixation

15 ml cacodylate buffer

5 ml stock 25% glutaraldehyde

0.5 ml 1% calcium chloride

2.12.9 1% Osmium tetroxide

13.4 mls dist. H_2O

6.6 mls 0.2m cacodylate buffer

0.2 mls 1% calcium chloride

0.2 gms osmium tetroxide (Agar Aids, 66a Cambridge Road, Stanstead, Essex CM24 8DA).

2.12.10 * Methanols (James Burrough Ltd., Fine Alcohols Division, 60 Montford Place, London SE11).

100% direct from bottle

90% 90 mls Methanol + 10 mls dist. H_2O

70% 70 mls Methanol + 30 mls dist. H_2O

60% 60 mls Methanol + 40 mls dist. H_2O

30% 30 mls Methanol + 70 mls dist. H_2O

* Must be A.R. Quality Methyl Alcohol CH_3OH

2.12.11. Cacodylate buffer (0.2m)

21.4 gms sodium cacodylate $Na(CH_3)_2AsO_2\ 3H_2O$ (Taab Laboratories)

3.45 ml N.HCl

Made up to 500 ml with dist. H_2O

2.12.12 Propylene oxide

1,2-Epoxypropane obtained from Taab Laboratories, 52, Kidmore End Road, Emmer Green, Reading.

2.12.13. Durcopan

Solution B-Hardene 100 mls

Solution A.M. Resin 80 mls

Solution D Plastissier 2 mls

Stir for 20 mins.

Solution C Accelerator 4 mls

Degas for 15 mins

Obtained from EMScope, Kingsnorth Industrial Estate, Wotton Road, Ashford, Kent TN23 2LN

2.12.14 PTA (Agar Aids, Stanstead, Essex CM24 8DD)

3% aqueous solution to pH 6.5 - 7.0 with 1N potassium hydroxide

2.12.15 Conjugates

Issued freeze dried in bottles each containing the residue from 1 millilitre of sterile conjugate in phosphate buffered saline containing 1% glucose and 0.1% sodium azide as preservative. Each bottle contains approximately 10 mg of protein with a minimum of 1 mg of specific Ab. prepared from hyperimmune antisera raised in rabbits or sheep against the appropriate purified IgG.

Supplied by: Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, Kent BR3 3BS.

2.12.16 R.P.M.I. 1640 Medium (Moore et al., 1967)

Imperial Laboratories, Ashley Road, Salisbury, Wiltshire

2.12.17 H.A.T. Medium (for monoclonal production)

Materials

H-Hypoxanthine (6-hydroxypurin M.wt. 136.1) 10×10^{-2}

(B.D.H. Chemicals Ltd., Poole, England)

A-Aminopterin (4-amino-folic acid; 4-aminopteroyl glutamic acid).

(M.wt. 440.4) 4.0×10^{-5}M.

(Sigma (London) Chemical Co. Ltd., Fancy Road, Poole, England).

T-Thymidine (M.wt. 242.2) 1.6×10^{-3}M

(B.D.H. Chemicals Ltd., Poole, England)

Procedure: 100 fold stock solution of hypoxanthine and thymidine

1. Dissolve 136.1 mg hypoxanthine and 38.8 mg thymidine 100 ml of distilled water.
2. Warm to 45-50°C to dissolve powdered reagents.
3. Sterilize by membrane filtration and store at -20°C in 2 ml aliquots.

100 fold concentrated stock solution of aminopterin

1. Add 1.76 mg aminopterin to 90ml distilled water.
2. Add 1M NaOH dropwise until aminopterin dissolves and titrate to neutrality with 1M HCl.
3. Adjust final volume to 100 ml with distilled water.
4. Sterilize by membrane filtration, dispense into 2 ml aliquots and store at -20°C.

H.A.T. medium

20 ml foetal bovine serum (inactivated 56°C for 30 minutes)

1 ml stock hypoxanthine/thymidine

1 ml stock aminopterin

1 ml glutamine (given final concentration 2 mM)

1 ml penicillin/streptomycin (final concentration 100 mg/100 iu)

76 ml R.P.M.I. 1640

2.12.18 β-Propiolactone (BPL) (Fluka AG, Glossop, Derbyshire)

Stock stored at -20°C until required, made up in distilled water at +4°C and used immediately.

2.12.19 Actinomycin D (Aldrich Chemical Co. Ltd., Gillingham, Dorset)

3. RESULTS

3.1. TISSUE CULTURE STUDIES WITH LGA 391 AND MOPEIA VIRUSES

3.1.1. Susceptibility of different cell lines

Different cell lines known to support arenavirus growth were infected with Lassa amd Mopeia viruses at a multiplicity of infection (M.O.I.) of 0.1. The preliminary study was to determine the ability of each cell line to support growth, the level of viral production, and to assess cytopathic effect and plaquing ability.

All the viruses produced a CPE in Vero and CV-1 cells, commencing at the 5th day p.i. when inoculated with LGA 391 and M152. The other Mopeia viruses did not show a CPE until 6-8 days p.i. (Table 4). In both cell lines onset of a cytopathic effect was demonstrated by small foci of necrotic cells progressing to extensive cellular degeneration resulting in total destruction. The 'L' cell exhibited no CPE with any of the viruses. In the case of BHK the CPE was difficult to assess, since these cells showed some variation in their ability to grow and be maintained in P.M.E.

In both Vero and CV-1 cells LGA 391 virus particles were detected by EM 2-3 days p.i. Mopeia particles were detected between 4-5 days p.i. Detection by EM of virus in L cells and BHK ranged from 3-5 days p.i. In all cell lines, viruses were detected by EM before the appearance of CPE. LGA 391 was demonstrated by IFA on day 1 p.i. in Vero and CV-1 cells while in L cells and BHK it was day 2 p.i. Detection of the Mopeia viruses by IFA in all cell lines tested ranged between 2-3 days p.i. (Table 4).

TABLE 4. EARLIEST DETECTION OF AFRICAN ARENAVIRUSES IN VARIOUS STATIONARY CELL LINES

Virus strain	Detection by	Vero	'L' cell	BHK	CV1
LGA 391	IFA	D1	D2	D2	D1
	CPE	D5	-ve	+/-	D5
	EM	D3	D3	D4	D2
20410	IFA	D2	D2	D3	D2
	CPE	D7	-ve	+/-	D5
	EM	D3	D3	D5	D3
148	IFA	D3	D3	D3	D2
	CPE	D6	-ve	+/-	D5
	EM	D5	D3	D4	D4
150	IFA	D3	D3	D3	D3
	CPE	D6	-ve	+/-	D5
	EM	D4	D4	D4	D5
152	IFA	D2	D2	D2	D2
	CPE	D5	-ve	+/-	D5
	EM	D4	D3	D4	D4
Z478	IFA	D3	D3	D3	D2
	CPE	D8	-ve	+/-	D5
	EM	D5	D5	D5	D4

D = days post infection

As the cells used in the high containment suite were grown in a closed system with no facility for incubating in a CO_2 atmosphere, all the cultures had to be grown in medium with a high buffering capacity. The most suitable medium found was L15 or PME. The latter was found to maintain cells for longer periods. Therefore all cells were grown in PME for consistency. In each cell line tested LGA 391 grew faster than all the Mopeia strains, peak titres being reached on day 3 p.i. in CV-1 and Vero cells; day 4 p.i. in L cells and day 6 p.i. in BHK. Mopeia viruses reached peak titres between days 4-6 p.i. in all the cell lines and were at least 10-fold lower than LGA 391 levels. Each of the viruses took longer to become established in BHK cells (Table 5).

TABLE 5. MEAN PEAK LEVELS OF EXTRACELLULAR VIRUS OBTAINED IN STATIONARY CELL CULTURES INFECTED WITH AFRICAN ARENAVIRUSES AT M.O.I. 0.1

	Vero	L cell	BHK	CV-1
LGA 391	8.0 D4	7.5 D5	8.0 D6	8.2 D3
M 20410	5.2 D4	6.5 D5	7.2 D8	7.0 D4
M 148	6.3 D5	7.0 D5	7.0 D5	6.8 D4
M 150	6.8 D4	7.0 D4	7.1 D6	6.8 D4
M 152	7.4 D5	8.0 D5	7.9 D6	7.0 D3
Z478	5.5 D4	7.5 D5	7.9 D8	7.2 D5

Expressed as $\log_{10}$ $TCID_{50}$/ml

D = days post infection

N.B. Each value represents the mean of 5 experiments

In roller bottles all the arenaviruses reached their peak levels in a shorter time and it was found that using L-15 gave marginally better levels of extracellular virus (Table 6).

TABLE 6. MEAN PEAK LEVELS OF EXTRACELLULAR VIRUS OBTAINED FROM CELLS GROWN IN ROLLER BOTTLES IN L-15 MEDIUM INFECTED WITH AN M.O.I. 0.1

	Vero	BHK	L cell	CV-1
LGA 391	8.2 D2	8.0 D6	9.7 D3	8.9 D2
M 20410	7.0 D3	N.D.	8.2 D4	7.0 D4
M 148	7.3 D4	N.D.	N.D.	N.D.
M 150	7.4 D3	N.D.	8.0 D3	8.2 D4
M 152	7.8 D2	8.0 D6	8.8 D4	8.5 D2
Z 478	7.5 D3	7.8 D4	8.5 D4	7.5 D4

Expressed as Log_{10} $TCID_{50}$/ml

D = days post infection

N.B. Each value represents the mean of 5 experiments

As a result of the preliminary data it was decided to use Vero cells for assay studies as they gave a consistent CPE and plaque appearance. A $TCID_{50}$ assay according to Reed-Muench was possible in microassay plates, which enabled a large number of assays to be carried out in limited and restricted laboratory conditions. Although the plaque assay was possible the logistics proved impossible to implement owing to the number of assays required. Vero cells were also chosen since the original pools were obtained from Vero cultures and it was thought that to stray from the original cell line would be unwise before basic characterisation was completed.

The L cells and CV-1 proved to be the most productive in roller bottles. As future biochemical studies required large quantities of virus, it was considered appropriate to use these cells as the base for viral purification. However, it was discovered that the L cell population used contained a latent virus considered to be an Orbivirus, hence it was discarded. Therefore the purification procedure proceeded with Vero or CV-1 cells.

3.1.2. Effects of concentration and purification on the viruses

Table 7 summarises the levels of virus after concentration and purification.

TABLE 7. INFECTIVITY DATA AT DIFFERENT STAGES DURING PURIFICATION

	Vero		L cell		CV-1	
Stages in purification	LGA	M150	LGA	M150	LGA	M150
Clarified tissue culture fluid	8.7	8.2	8.7	7.0	8.0	ND*
Polyethylene glycol	10.5	9.8	10.5	7.7	9.6	ND
Glycerol-tartrate bands	11.0	10.1	11.0	9.7	10.0	ND
Glycerol-tartrate pellets	11.3	10.7	11.0	9.8	11.3	ND

Expressed as Log_{10} $TCID_{50}$/ml

ND - Not done

3.1.3. Growth kinetics of Lassa and Mopeia in Vero cells

3.1.3.1. Rate of Virus Adsorption

Vero cell monolayers in drained 25 cm^2 flasks were inoculated with approximately 100 plaque forming units (pfu) of virus in 0.2 ml aliquots. These were incubated at 37°C with intermittent rocking of the flasks to prevent drying of the cells and to improve distribution of virus seed. At various intervals bottles were removed from the incubator, washed and overlaid with 2% agarose in PME.

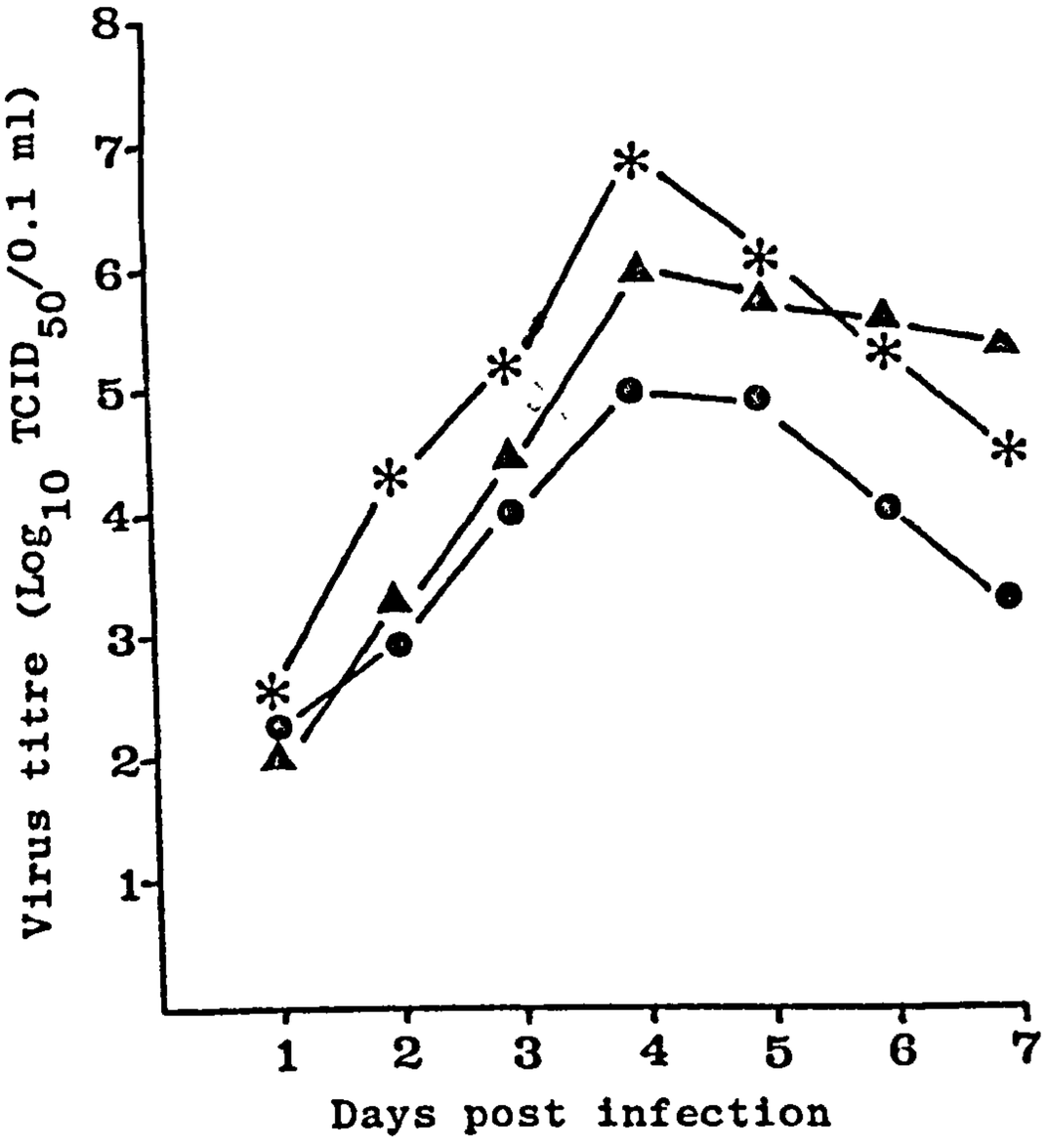
In all cases between 30-40% of the virus was absorbed within 30 minutes maximum adsorption being achieved within 120 minutes at 37°C.

3.1.3.2. Effect of different multiplicities of infection

Flasks (25 cm^2) containing Vero cell monolayers were inoculated at different MOIs. They were adsorbed for 2 hours at 37°C, washed, and 5 mls of PME containing 2% FCS added. Incubation was carried out at 37°C. Aliquots of 0.4 mls were removed daily, centrifuged at 1000 rpm to remove debris and titrated in microtitre plates containing Vero cell monolayers.

Figure 16 demonstrates the growth kinetics of LGA 391 and M20410 at different multiplicities of infection. A similar pattern was observed with the other Mopeia strains. It was evident that high multiplicities of infection (10 - 0.1) gave rise to low levels of extracellular virus production (10^3 - 10^4 $TCID_{50}$/ml). Low multiplicities of infection as demonstrated in Figures 16 and 17 provide the highest rates of growth during the first two to three days, maximum viral titre being achieved between 3-5 days. There was increased evidence of prozoning as measured by CPE. This was particularly evident in cultures containing high concentrations of virus over 3 days post infection and was a phenomenon common to all the strains studied. The degree of prozoning increased as the age of the cultures advanced.

(a)



(b)

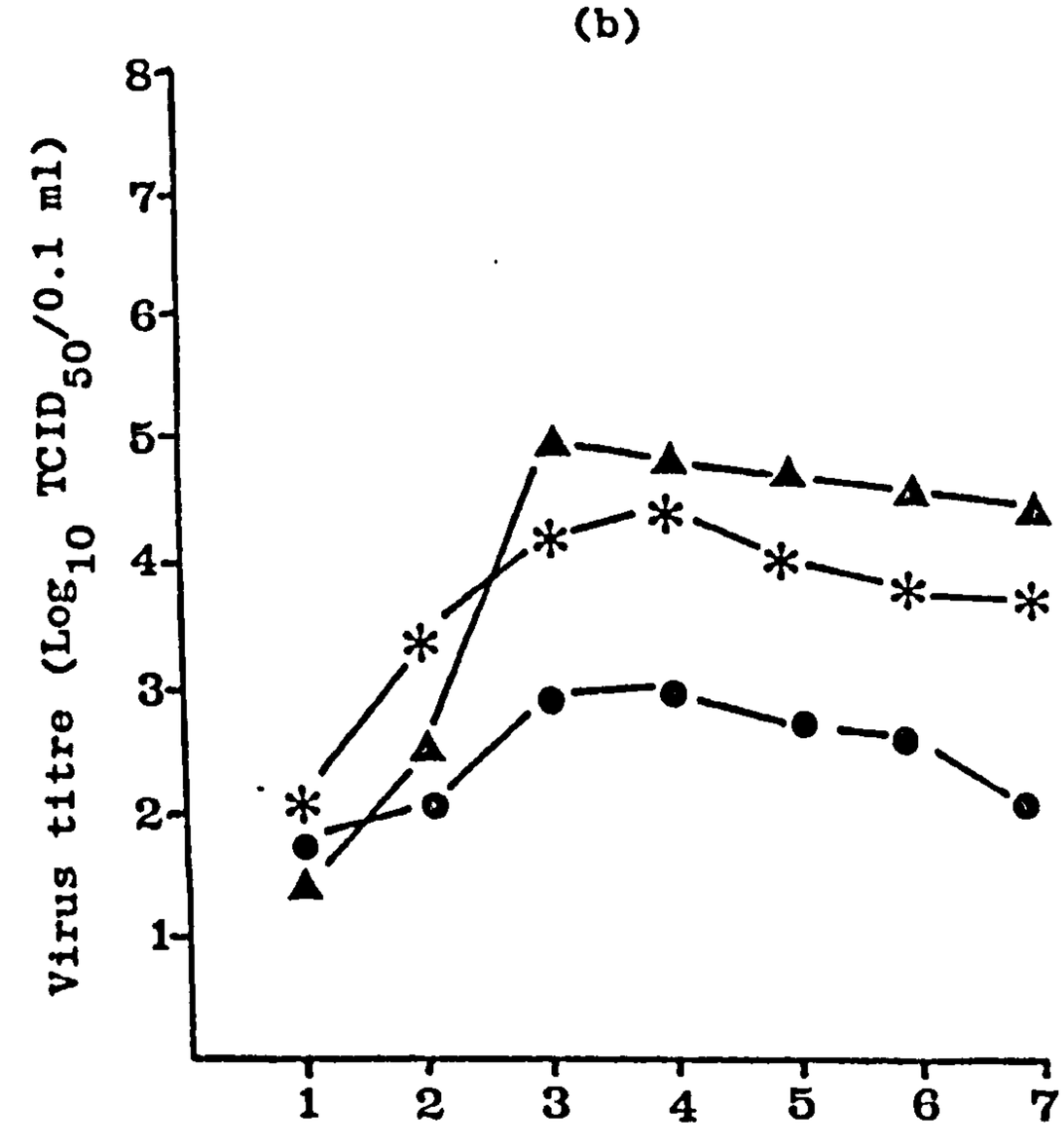


Fig.16 Growth kinetics of (a) LGA 391 and (b) M20410 in Vero cells at different multiplicities of infection in PME supplemented with 2% FCS at 37°C stationary cultures.

▲ M.O.I. 0.01 * M.O.I. 0.1 ● M.O.I. 1.0

Each point represents the geometric mean of 5 experiments.

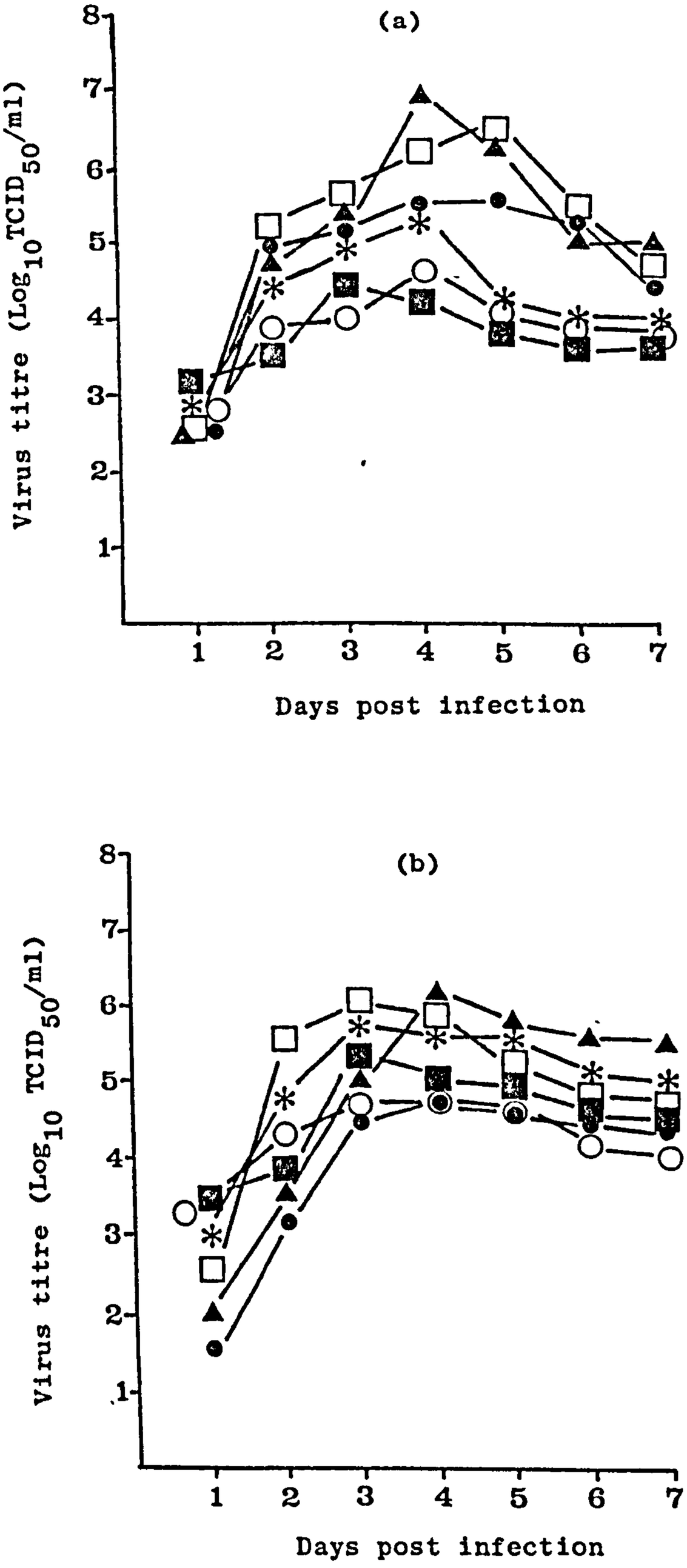


Fig.17 Growth kinetics of Lassa and Mopeia viruses in Vero cells at different multiplicities of infection (M.O.I.) (a) 0.1, (b) 0.01.

▲LGA 391 □M152 ✳M150 ●M148
○M20410 ▩Z478

Each point represents the geometric mean of 5 different experiments

3.1.3. Growth kinetics of Lassa and Mopeia in Vero cells

3.1.3.1. Rate of Virus Adsorption

Vero cell monolayers in drained 25 cm^2 flasks were inoculated with approximately 100 plaque forming units (pfu) of virus in 0.2 ml aliquots. These were incubated at 37°C with intermittent rocking of the flasks to prevent drying of the cells and to improve distribution of virus seed. At various intervals bottles were removed from the incubator, washed and overlaid with 2% agarose in PME.

In all cases between 30-40% of the virus was absorbed within 30 minutes maximum adsorption being achieved within 120 minutes at 37°C.

3.1.3.2. Effect of different multiplicities of infection

Flasks (25 cm^2) containing Vero cell monolayers were inoculated at different MOIs. They were adsorbed for 2 hours at 37°C, washed, and 5 mls of PME containing 2% FCS added. Incubation was carried out at 37°C. Aliquots of 0.4 mls were removed daily, centrifuged at 1000 rpm to remove debris and titrated in microtitre plates containing Vero cell monolayers.

Figure 16 demonstrates the growth kinetics of LGA 391 and M20410 at different multiplicities of infection. A similar pattern was observed with the other Mopeia viruses. It was evident that high multiplicities of infection (10 - 0.1) gave rise to low levels of extracellular virus production (10^3 - 10^4 $TCID_{50}$/ml). Low multiplicities of infection as demonstrated in Figures 16 and 17 provide the highest rates of growth during the first two to three days, maximum viral titre being achieved between 3-5 days. There was increased evidence of prozoning as measured by CPE. This was particularly evident in cultures containing high concentrations of virus over 3 days post infection and was a phenomenon common to all the strains studied. The degree of prozoning increased as the age of the cultures advanced.

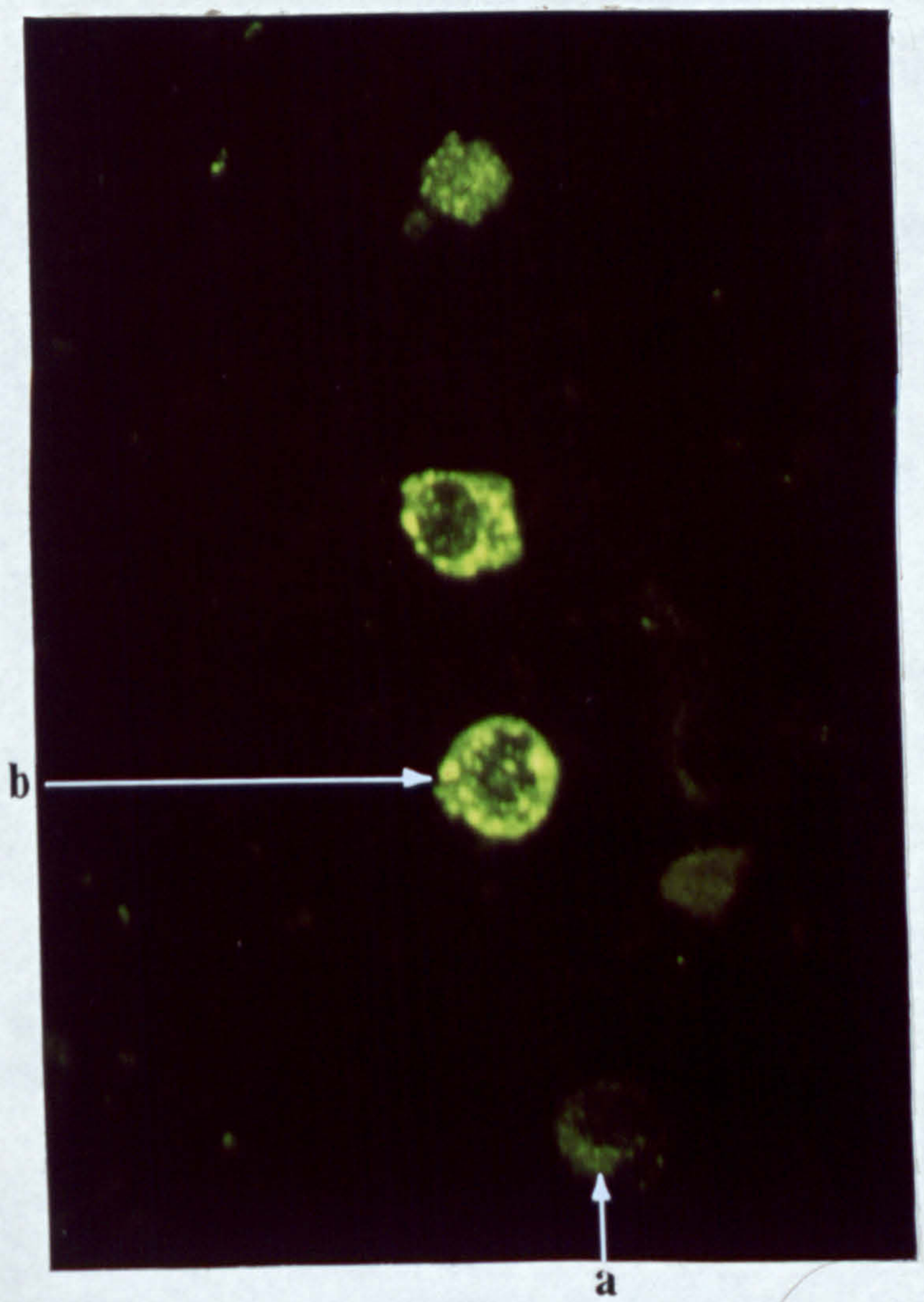


Fig.18 A photograph of LGA391 fluorescence in vero cells showing (a) early fine granular cytoplasmic inclusions and (b) the latter stages of large cytoplasmic inclusions in close proximity to the cytoplasmic membrane. (x400).

Examination by IFA of fixed and unfixed Vero cell cultures infected with Lassa or Mopeia viruses using guinea pig anti-arenavirus hyperimmune sera or Lassa and Mopeia monoclonal antibodies revealed similar cellular changes during infection. An identical pattern of events occurred with each virus studied as outlined below.

Following infection of cells with MOI's of 0.1 or 0.01, detection of LGA 391 and Mopeia viruses by IFA ranged from day 1 to day 3 dependent on the reagents used. The presence of virus was observed by day 1-2, which was earlier than observable CPE (Table 4). Fluorescence in the early stages of infection was denoted by a fine granular cytoplasm which progressed during the latter stages to display large cytoplasm inclusions in close proximity to the cytoplasmic membrane (Fig. 18). No observable nuclear involvement was noted. Experience using monoclonal antibodies has demonstrated how valuable these reagents are in detecting viral antigens earlier than the hyperimmune sera by virtue of the fact that less non-specific responses are likely to occur.

3.1.4. Growth kinetics of Lassa and Mopeia viruses in L cells

Maximum adsorption for all the viruses in stationary and roller bottles was achieved within 120 minutes at 37°C by the method outlined for Vero cells (Section 3.1.3.1.).

The maximum production of extracellular virus in stationary or roller bottles was dependent on the multiplicity of infection. Figure 19 demonstrated the growth kinetics of LGA391 with varying M.O.I.s. Similar results were achieved with all the strains of Mopeia viruses. High M.O.I.s ranging between 1 to 10 resulted in lower levels of virus production (2-5 $\log_{10}$ $TCID_{50}$/ml) and were accompanied by the demonstration of prozoning in micro-assay tissue culture titrations. Samples had to be diluted 10^{-3} to eliminate the prozoning in culture. Low M.O.I.s were found to be necessary for the production of high extracellular virus with either Lassa or Mopeia viruses.

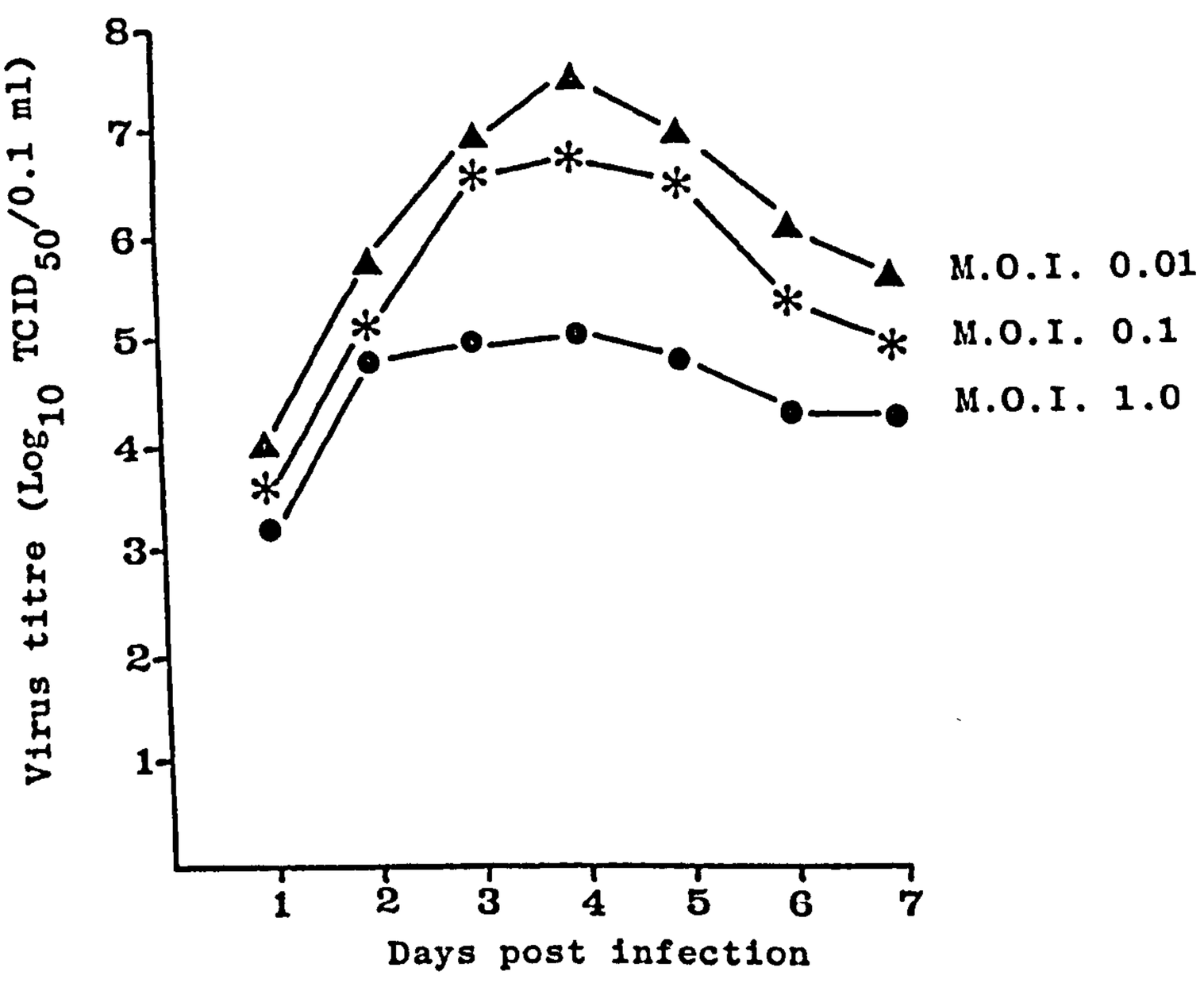


Fig. 19 Growth kinetics of LGA 391 in 'L' cells at different multiplicities of infection in PME supplemented with 2% FCS at 37°C stationary cultures.

Each point represents the geometric mean of 5 experiments.

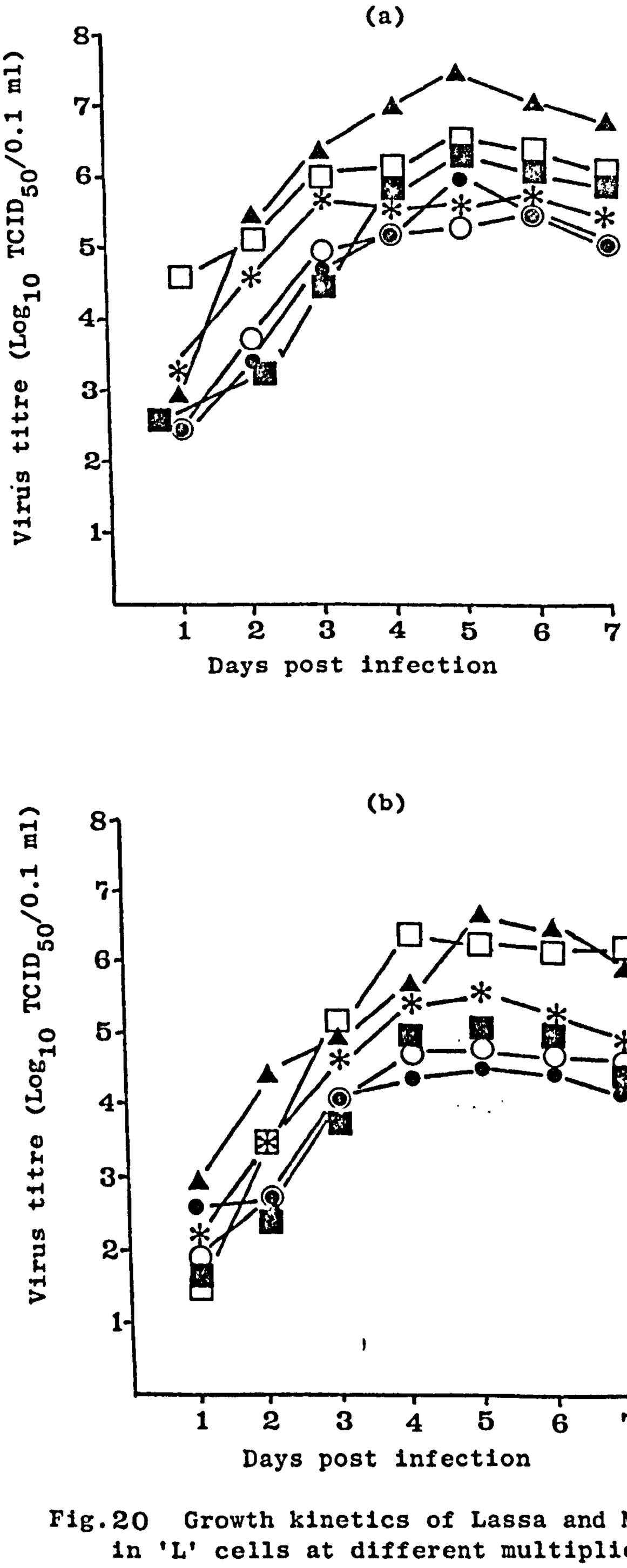


Fig.20 Growth kinetics of Lassa and Mopeia viruses in 'L' cells at different multiplicities of infection (M.O.I.) (a) 0.1, (b) 0.01.

▲LGA 391 □M152 ✱M150 ●M148 ○M20410
▣Z478

Each point represents the geometric mean of 5 different experiments.

The growth kinetics of LGA391 and Mopeia viruses in 'L' cells at M.O.Is of 0.1 or 0.01 are shown in Fig. 20a and b. Maximum yields of viruses occurred between 3-5 days p.i.; thereafter a gradual reduction was noted. A more rapid growth of Lassa and Mopeia strains was observed with an M.O.I. of 0.1. L cells inoculated with LGA391 and M152 viruses produced the highest levels of virus at both 0.1 and 0.01 M.O.I. The other strains of Mopeia virus produced maximum yields within the same time (day 3-5) but always 10^1 -10^2 $TCID_{50}$/ml below that achieved with LGA 391 and M152.

None of the Lassa or Mopeia viruses initiated a C.P.E. in 'L' cells, although it was noted that during viral growth the cells were easily detached from the surface of the flasks.

Examination of the cells histologically or by IFA demonstrated a similar developmental pattern as found in Vero cells.

3.1.5. Growth kinetics of Lassa and Mopeia viruses in CV-1 cells

Maximum adsorption for all the viruses in stationary and roller bottles was achieved within 120 minutes at 37°C by the method outlined for Vero cells (Section 3.1.3.1.).

The production of extracellular virus to peak levels is less dependent on the level of M.O.I. Figure 21 demonstrates the growth kinetics of LGA391 with varying M.O.I.s. Similar results were achieved with all the strains of Mopeia viruses. With M.O.I.s of between 0.01 and 1.0 maximum yields of virus were achieved by day 2.

With an M.O.I of 0.1 or 0.01 a consistent pattern of growth kinetics was observed with Lassa and Mopeia viruses. Peak levels of extracellular virus were observed by day 2 with LGA 391, M152, M150 and Z478 and by day 3 with M20410 and Z478 (Figs. 21 and 22). There were no major differences in the levels of virus production between the two M.O.I.s except that the initial growth phase was one day longer when using a M.O.I. of 0.01. As with Vero and L cells LGA 391, M150 and Z478 were the most

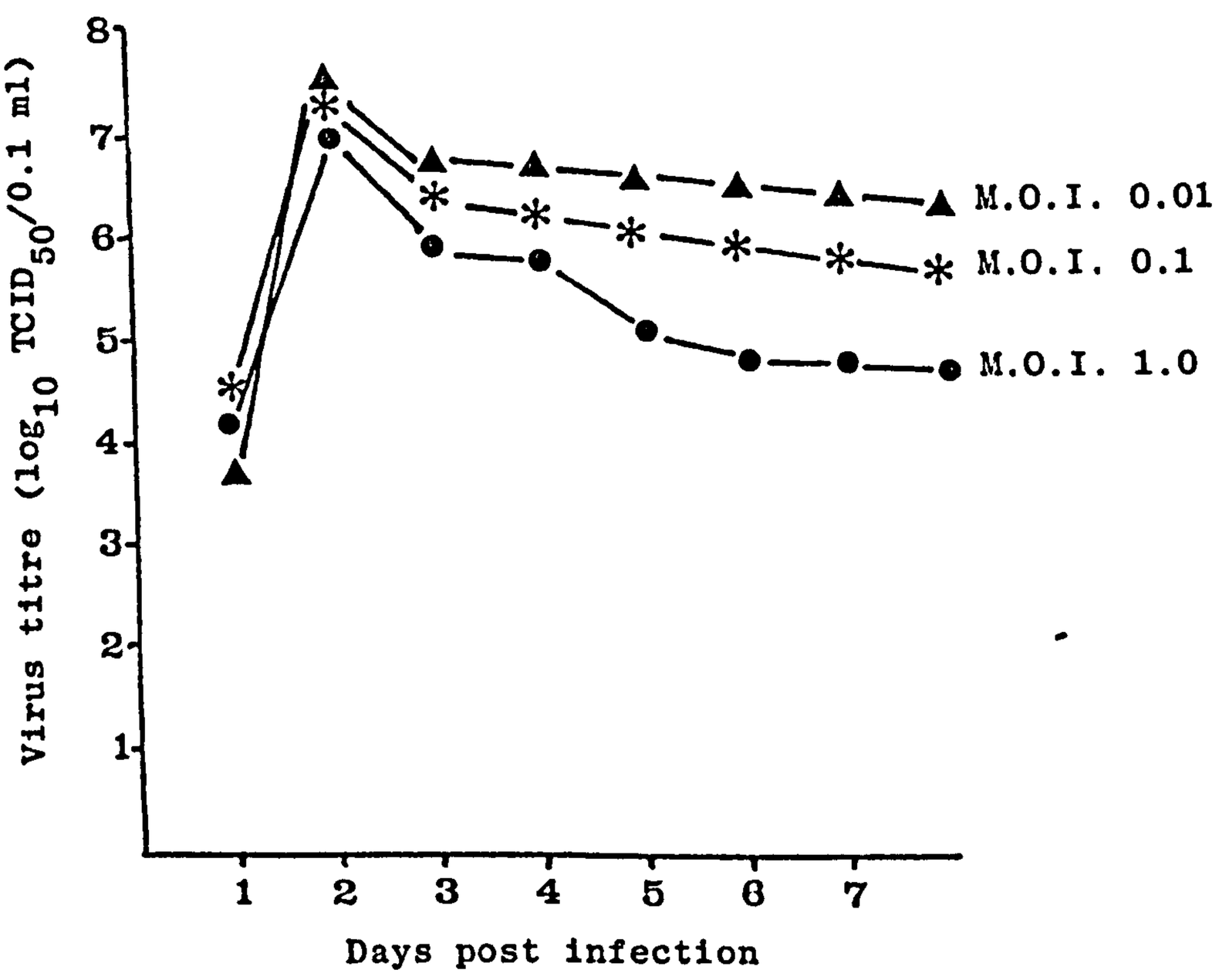


Fig.21 Growth kinetics of LGA 391 in CV-1 cells at different multiplicities of infection in PME supplemented with 2% FCS at 37°C stationary cultures.

Each point represents the geometric mean of 5 experiments.

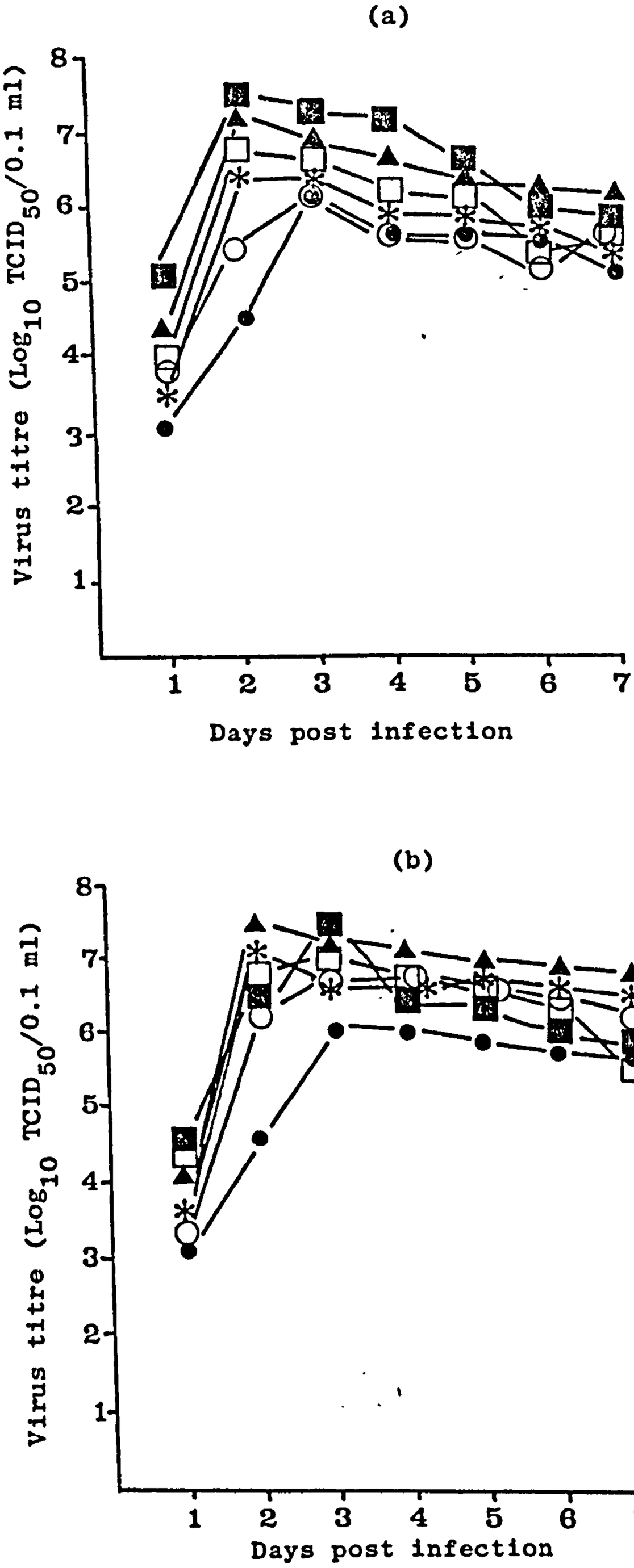


Fig.22 Growth kinetics of Lassa and Mopeia viruses in CV-1 cells at different multiplicities of infection (M.O.I.) (a) 0.1, (b) 0.01.

▲ LGA 391 □ M152 ✻ M150 ● M148

○ M20410 ■ Z478

Each point represents the geometric mean of 5 experiments.

productive achieving between 10^1 - 10^2 $TCID_{50}$/0.1 ml higher levels than the other strains (Fig. 22). Prozoning was observed in low dilutions (10^{-1}) from samples tested after day 1.

The cytopathic effect was evident with all the viruses on day 5. It appeared more rapidly than in Vero cells. There was no early rounding of the cells with the accompanying granulation. The CPE was rapid and uniform extending over the whole culture, resulting in extensive detachments of the affected cells.

Examination by cellular histology or by IFA demonstrated a similar developmental pattern as found in Vero cells.

3.2. ELECTRON MICROSCOPIC STUDIES OF TISSUE CULTURE MATERIAL

3.2.1. Morphology of virus particles

The morphological details of the Mopeia viruses were identical with that of Lassa. Identification on sub-grouping of the individual viruses was not possible on morphological grounds. The morphological structure of the virus particles and their development in cell culture or animal tissue are similar so the following general description applies to all the viruses studied even though only limited examples are shown (Figs. 23-42).

In ultra-thin sections Lassa and Mopeia virus particles appeared round or oval with varying degrees of pleomorphism. The mean diameters of the particles showed minor differences (Table 8).

TABLE 8. MEASUREMENT OF AFRICAN ARENAVIRUSES

Virus	Mean Diameter (nm)	Individual variation (nm)
M 20410	140	130-150
M 148	170	80-300
M 150	120	100-150
M 152	120	100-240
Z 478	160	120-230
Z 481	200	150-220
LGA 391	160	80-250

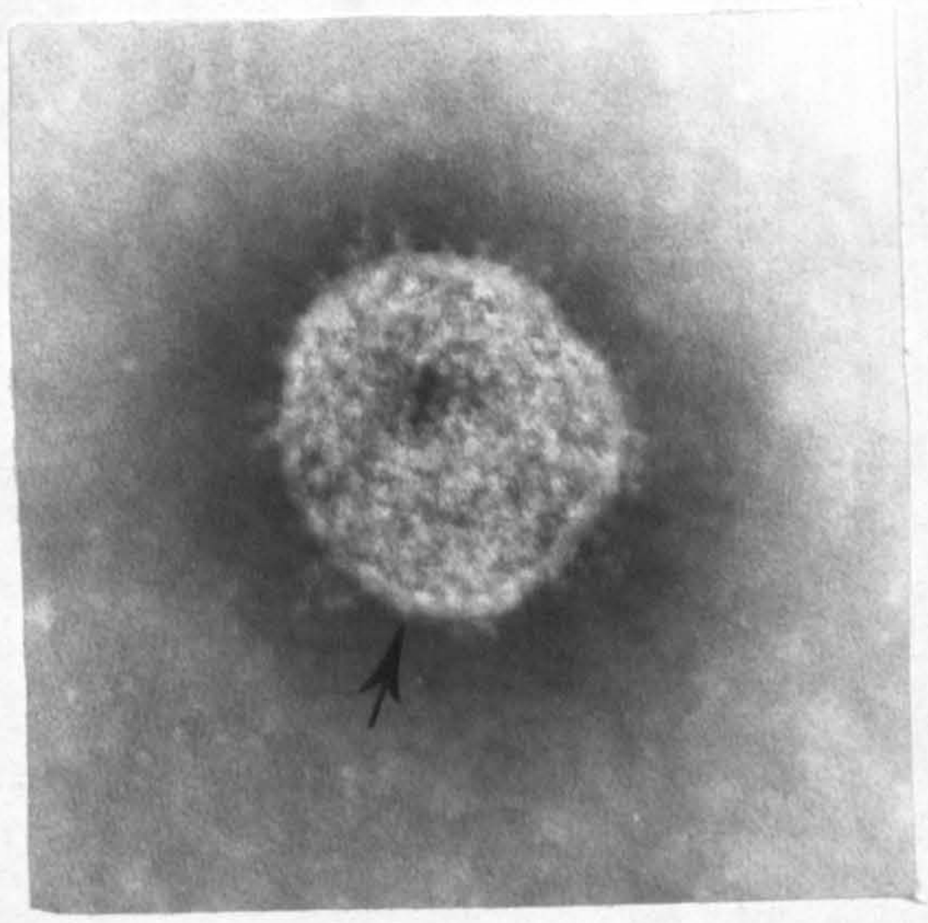
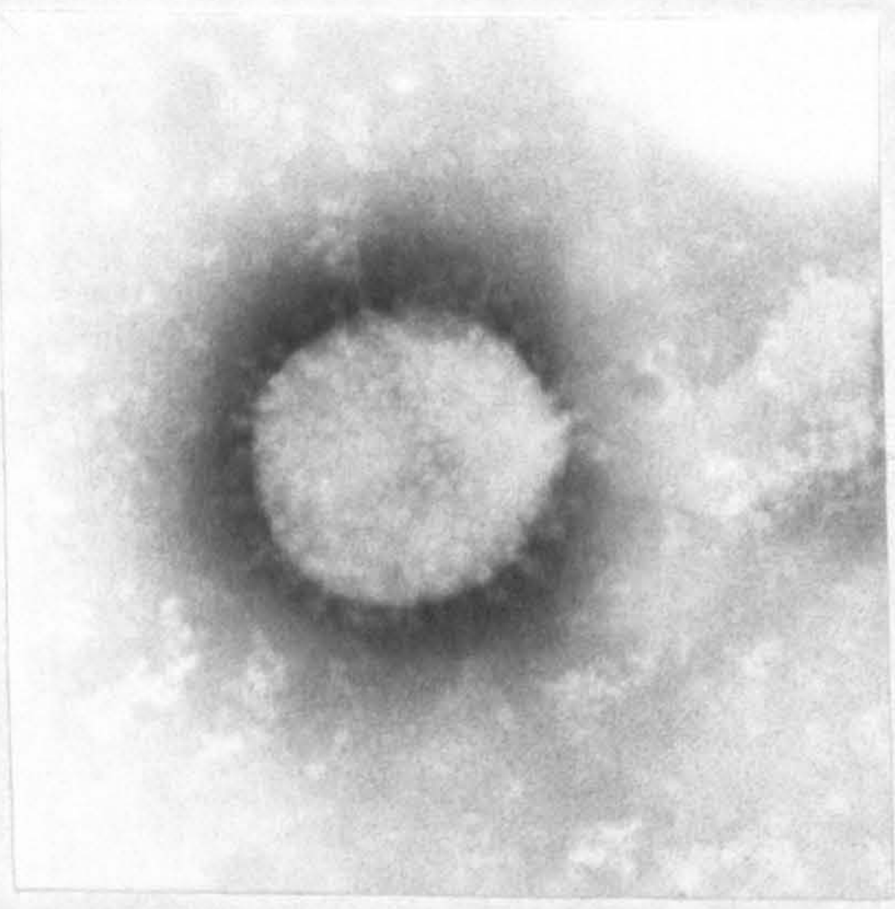


Fig.23
M150 virus particle which has not been completely penetrated by negative contrast medium revealing membrane nature of its envelope (arrow). Surface projections more widely spaced than other Mopeia particles.

x 200,000

Fig.24
M20410 virus particle showing typical arenavirus morphology.

x 200,000



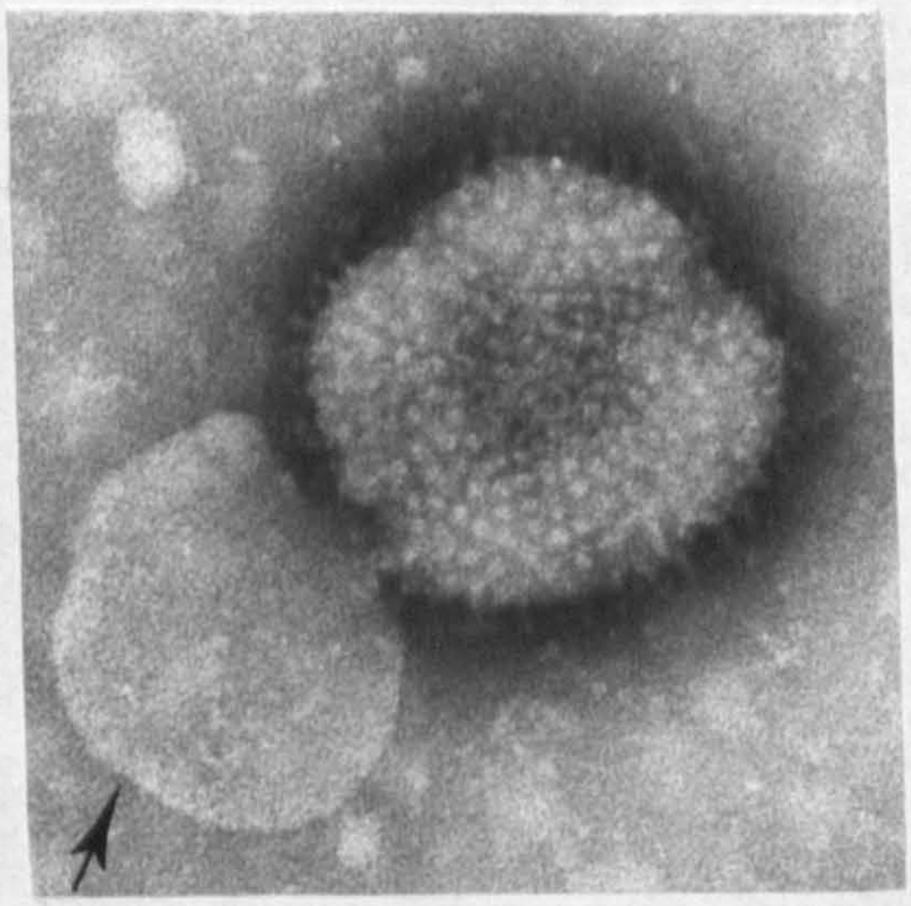


Fig.25
Z478 virus particle showing typical arenavirus morphology. Also demonstrates a membranous bleb (arrow).

x 200,000

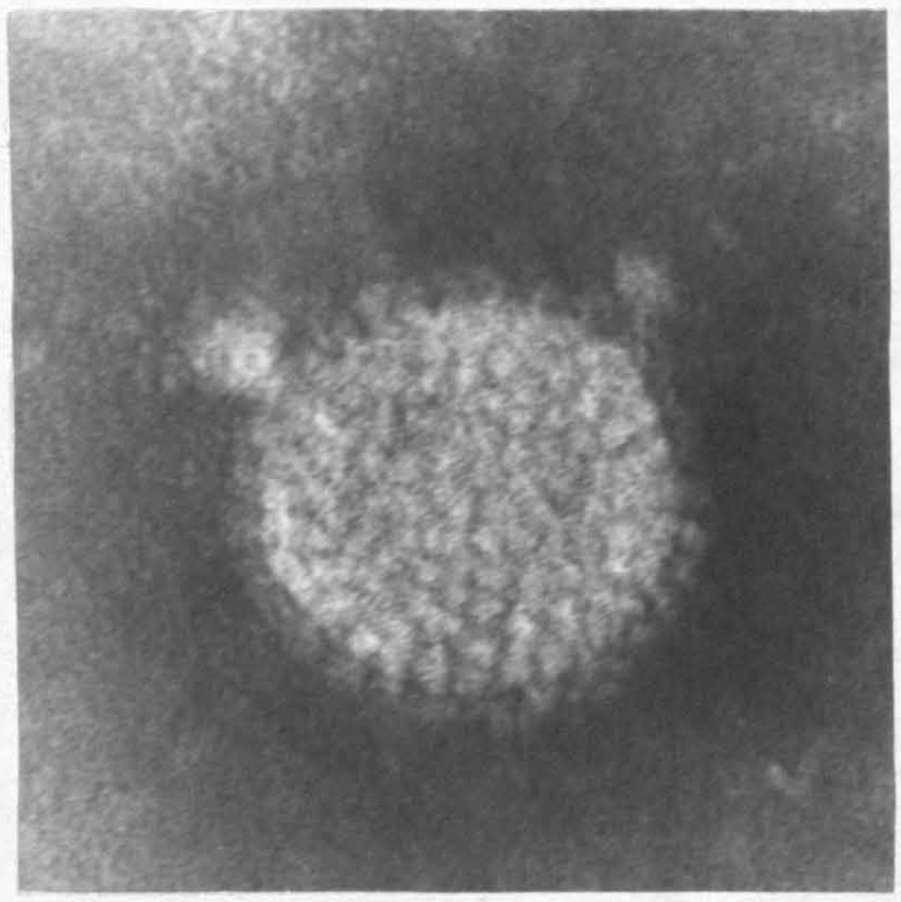


Fig.26
LGA 391 virus particle in negative contrast, showing typical granular appearance and closely spaced surface projections.

x 176,000

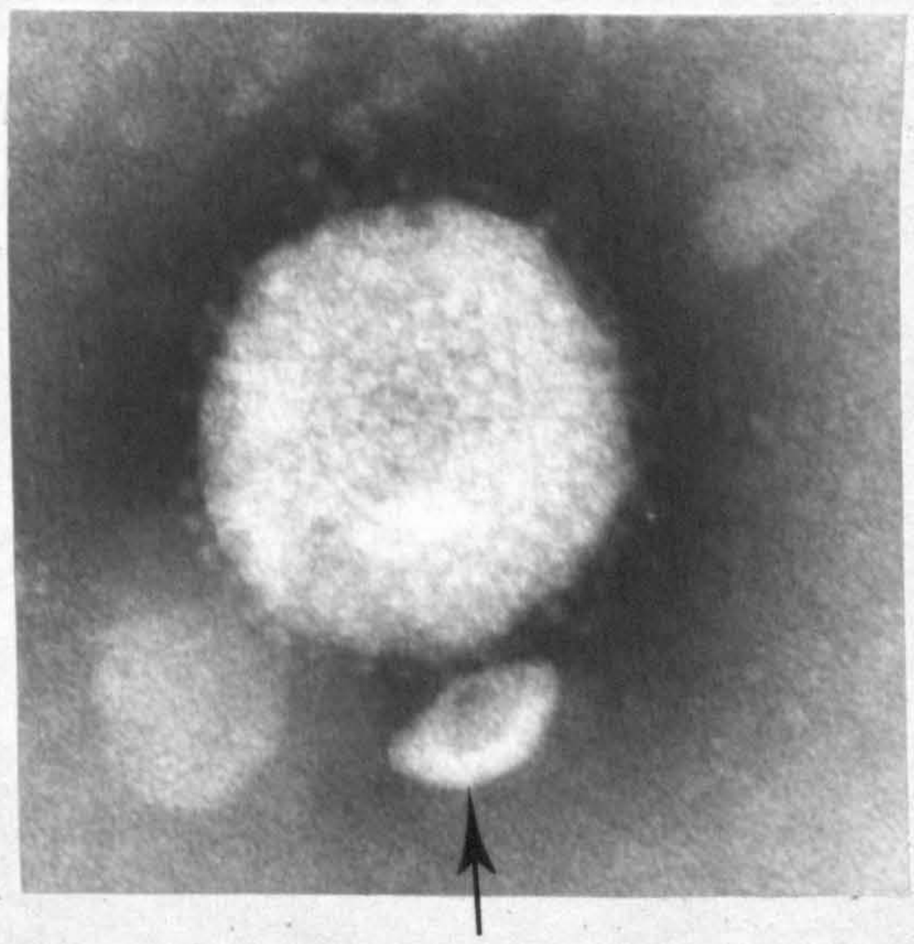


Fig.27
M148 virus particle showing typical granular appearance. Delicate membranous bleb (arrow) was considered to result from osmotical sensitive envelope membrane. Surface projections separated more widely.

x 200,000

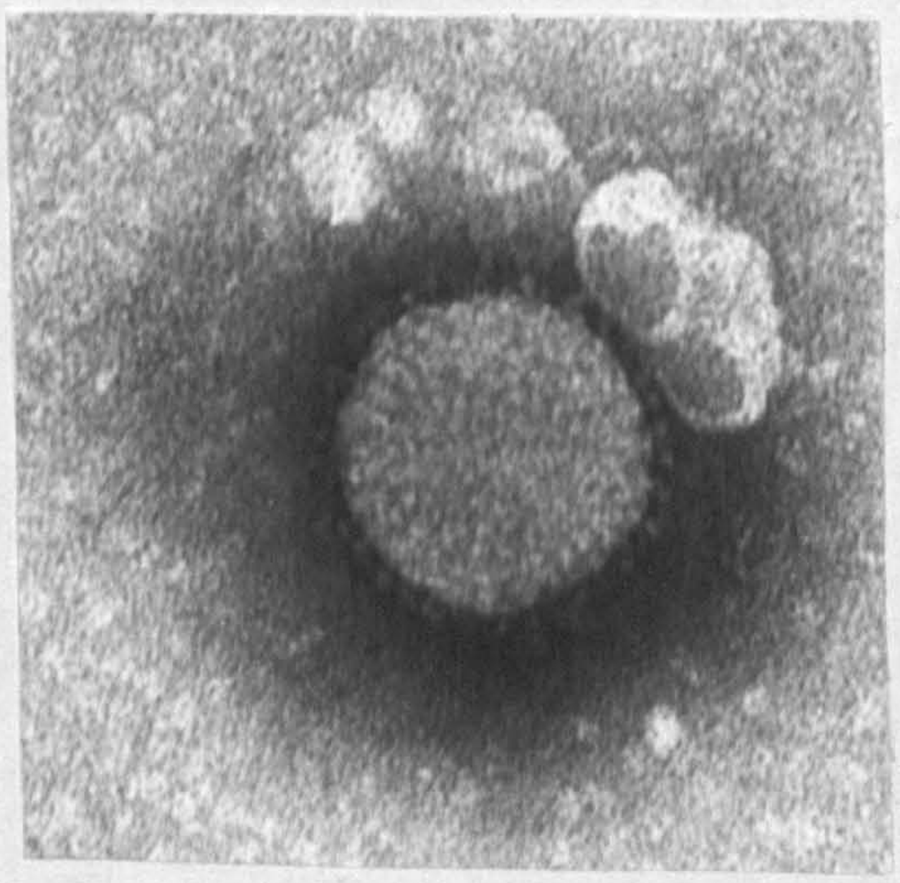


Fig.28
M152 indistinguishable from other arenavirus particles. Uniform granular appearance with closely spaced surface projections.

x 200,000

The morphology of LGA 39 and Mopeia viruses differs little from the standard arenavirus particle. All the viruses demonstrated surface projections forming an outer layer having a regular configuration, reflecting the shape and spacing of the individual projections. The viral envelopes were formed from the basic trilayer of the plasma membrane of the host cell, which were modified by a significant increase in the width of both membrane leaflets and by an increase in the width and uniformity of the electron dense intermediary zone. This envelope layer may take on a more complex form. The internal structure of all the viruses has been shown to contain varying numbers of 20-30 nm electron dense particles, which were easily distinguishable from glycogen granules, but were morphologically indistinguishable from ribosomes (Figs. 29-42). The distribution of these particles appeared to be random although there were indications that there are linear structures connecting the ribosomes.

Negative contrast microscopy of the viruses demonstrated the friability of the unit membrane envelope of the particles giving rise to bizarre shapes. In addition the staining and drying conditions often caused osmotic blebbing of the membrane. It was also noted that to prevent complete distortion of these particles there was a critical pH range for the staining of each particle (Table 9). It must also be noted that the procedure for removal of the particles out of a High Containment Laboratory may also have some bearing on the quality of these studies, a factor beyond the control of the author.

TABLE 9. PH TOLERANCE DURING NEGATIVE STAINING

Virus	pH range
LGA 391	6.8 - 7.0
M 20410	6.2 - 6.5
M 148	6.2 - 6.6
M 150	6.2 - 6.4
M 152	6.2 - 6.5
Z 478	6.2 - 6.4
Z 481	6.2 - 6.4

The surface projections have been shown to be closely arranged on all the viruses, but osmotic swelling can have an effect on these special arrangements. The individual projections do have a degree of variation in their size (Table 10) even though they conform to the recognizable club shape appearance.

TABLE 10. COMPARATIVE SIZES OF PROJECTIONS ON THE SURFACE FOR AFRICAN ARENAVIRUSES

Viruses	Mean size range (nm)
LGA 391	15-21
M 20410	8-10
M 148	10-15
M 150	8-10
M 152	15-20
Z 478	8-13
Z 481	8-12

3.2.2. Viral morphogenesis and infected cell characteristics

All the Mopeia and Lassa viruses grown in Vero, CV-1, LLC-Mk2, BHK and L-cells mature by budding from the plasma membrane (Figs. 29-42). During the early part of the infection the host cell membrane changes at bud sites which indicates the onset of viral envelope construction (Figs. 29, 32, 34, 37, 40). Late in infection areas of the plasma membrane became more dense, and had observable surface projections (Figs. 30, 33-35, 37, 39). Distinctive intracytoplasmic inclusion bodies were found (Figs. 34, 38, 41). The inclusions consisted of an electron dense, smooth matrix in which the dense particles were identical with those found in the virus particles. As illustrated the granules were homogeneous in size and were indistinguishable from host-cell ribosomes. In all cases the inclusions varied in their degree of condensation, were not confined within structural margins and varied widely in shape and size. Infection of Vero, LLC-Mk2, CV-1 and BHK cells with all the African arenaviruses studied causes

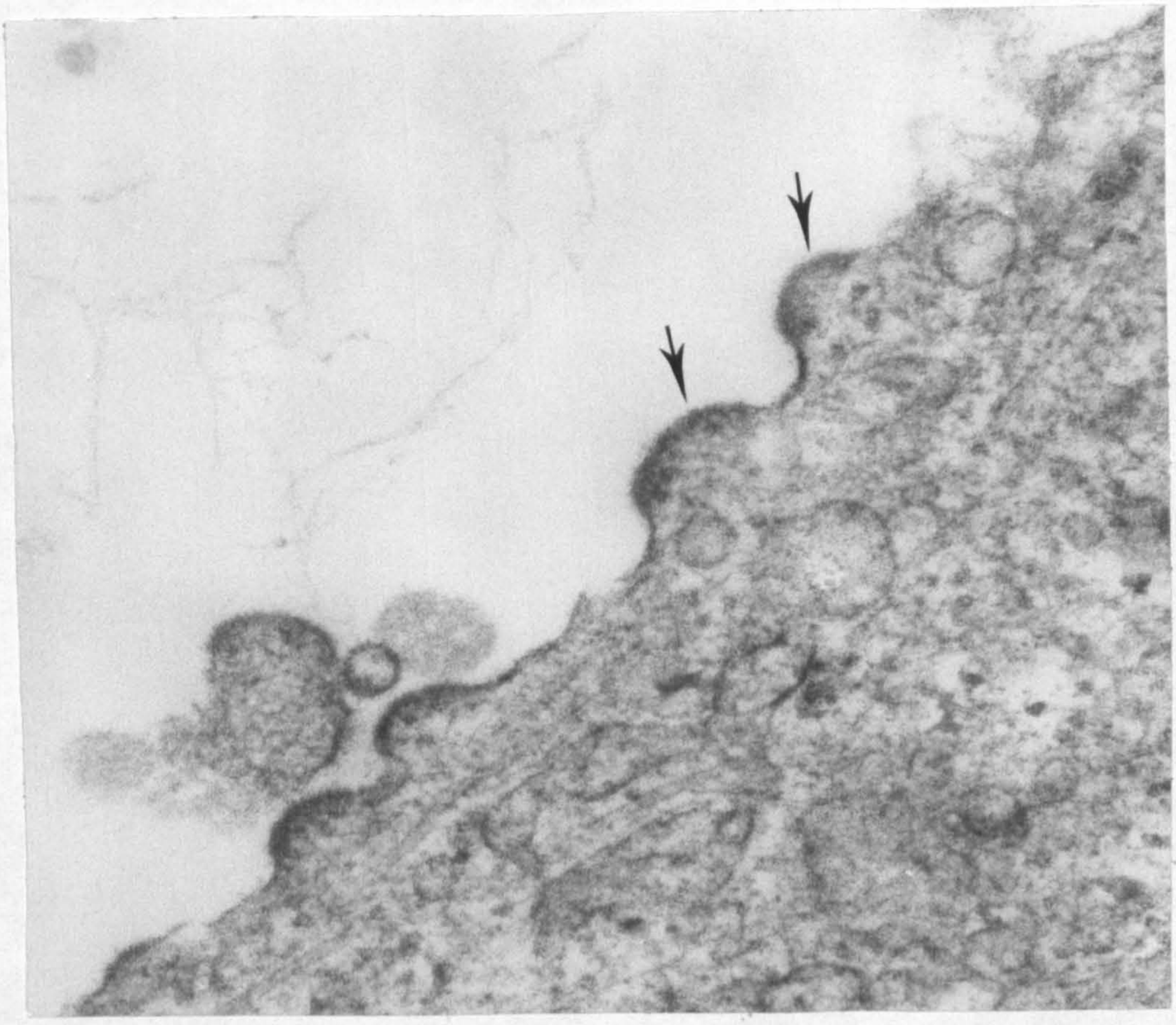


Fig.29. LGA 391 virus infected Vero cell (day 2 p.i.) with particles budding from plasma membrane (arrows). Envelope structure more distinct than normal plasma membrane at sites of particle budding. (x 50,000)

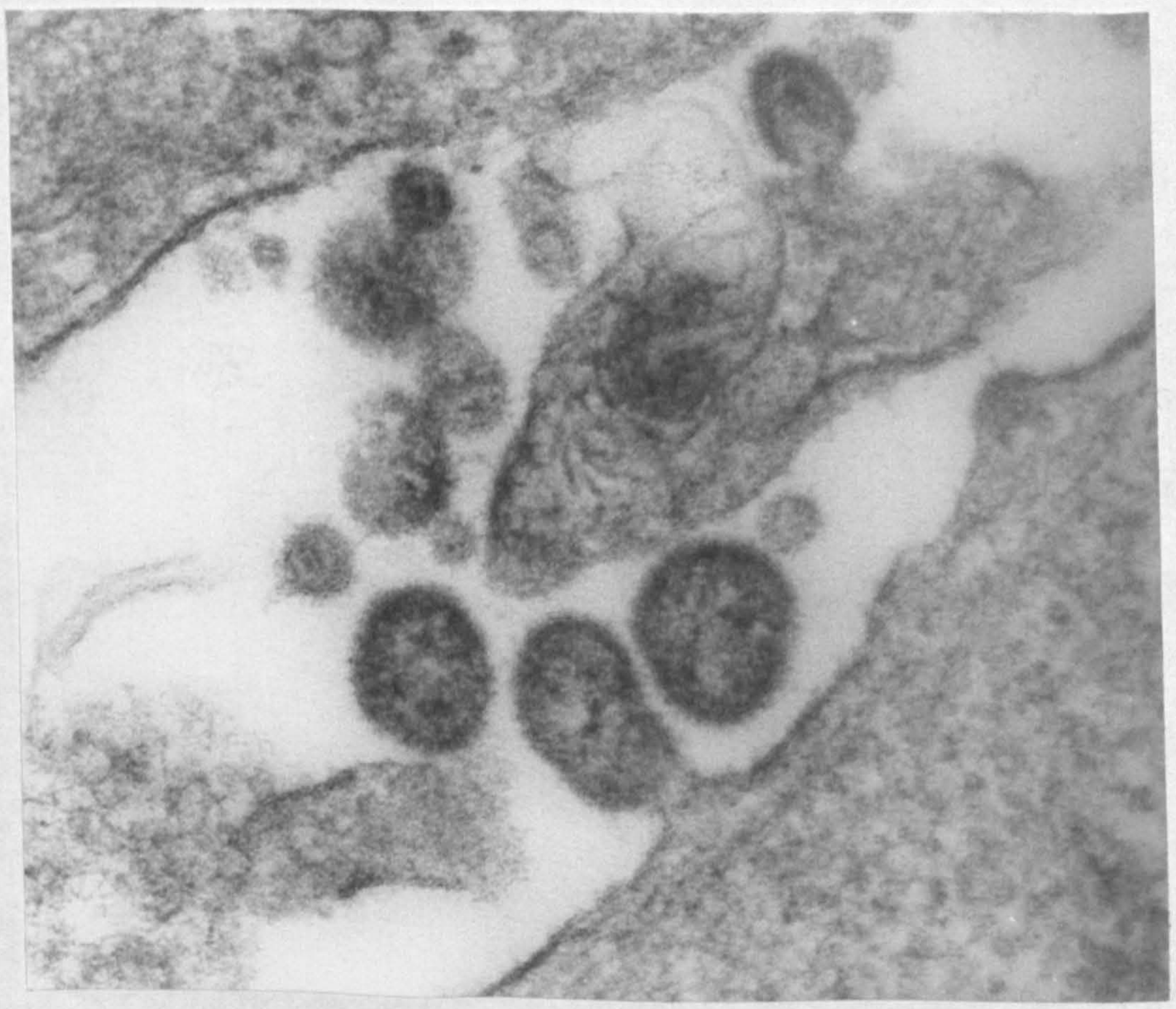


Fig.30. LGA 391 virus particles in final stages of budding from Vero cell surface (day 5 p.i.). (x 50,000)

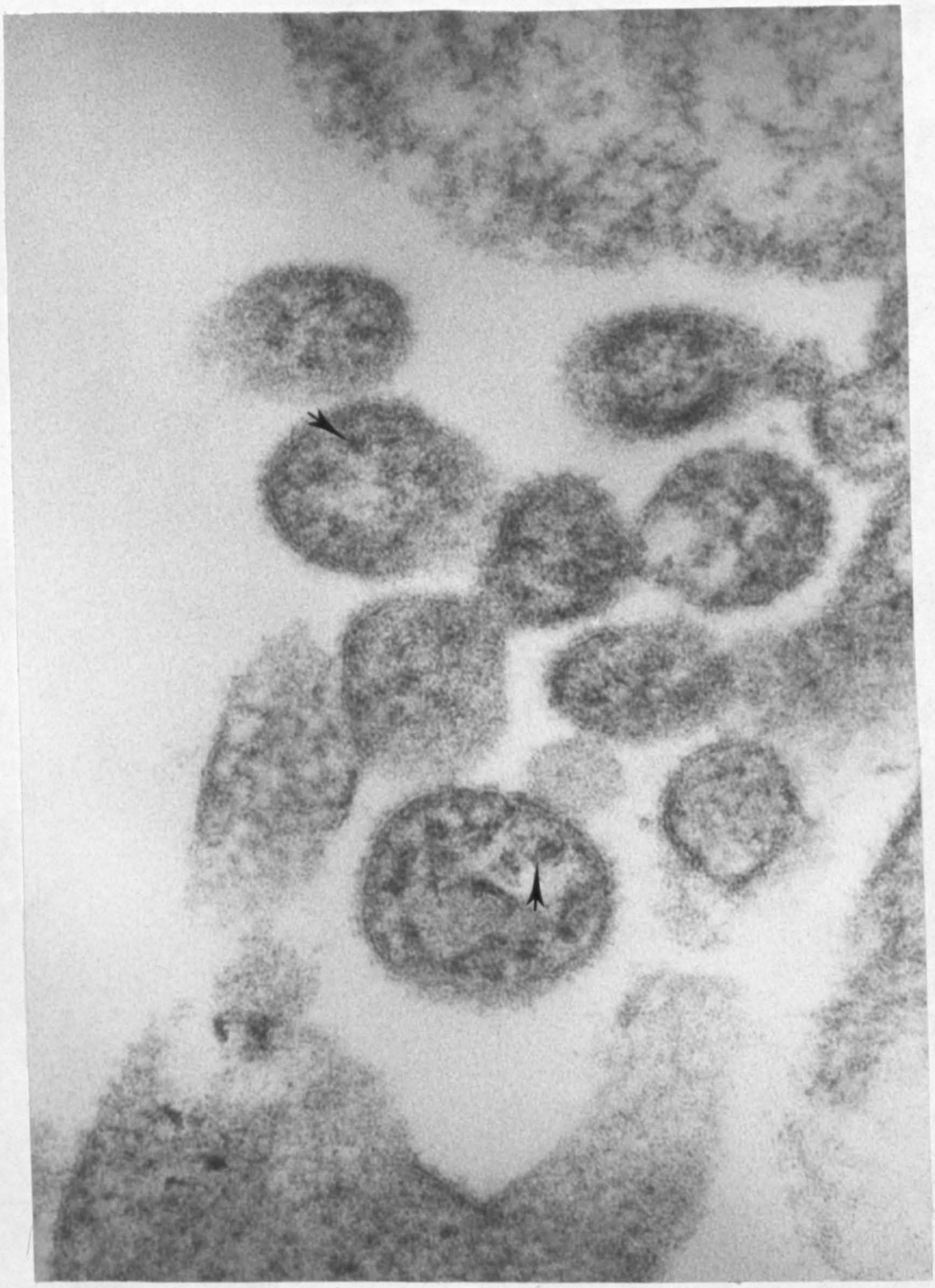


Fig.31. LGA 391 virus particles in extracellular space (day 5 p.i.) at the periphery of Vero cells. Particles consist of heavy membrane envelope containing various amounts of 20 to 25 nm dense granules (arrows). (x 200,000)

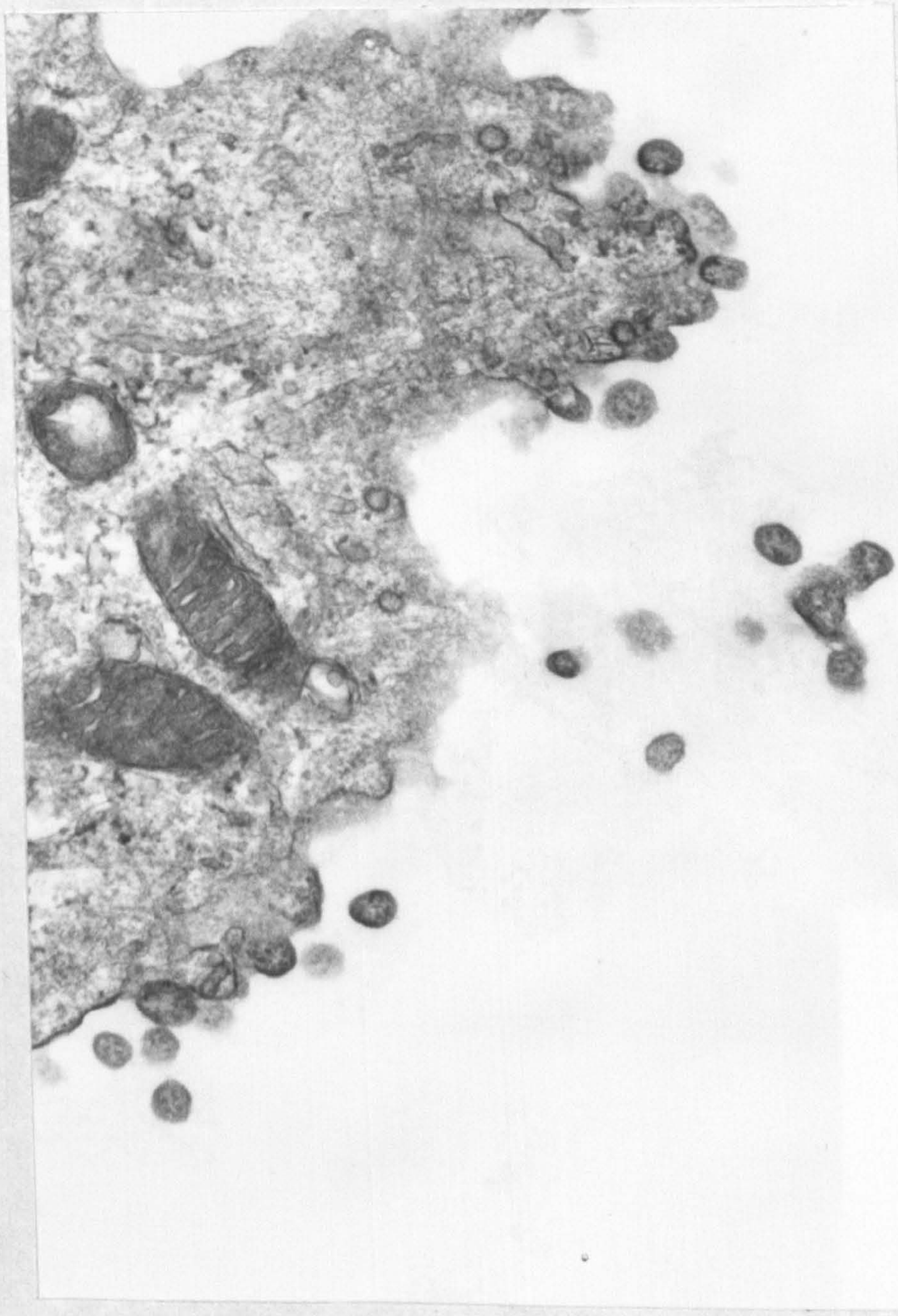


Fig.32. M148 virus infected Vero cell (day 4 p.i.) with particles budding from plasma membrane and free in extracellular space. Envelope structure more distinct than normal plasma membrane at sites of potential particle budding. (x 40,000)

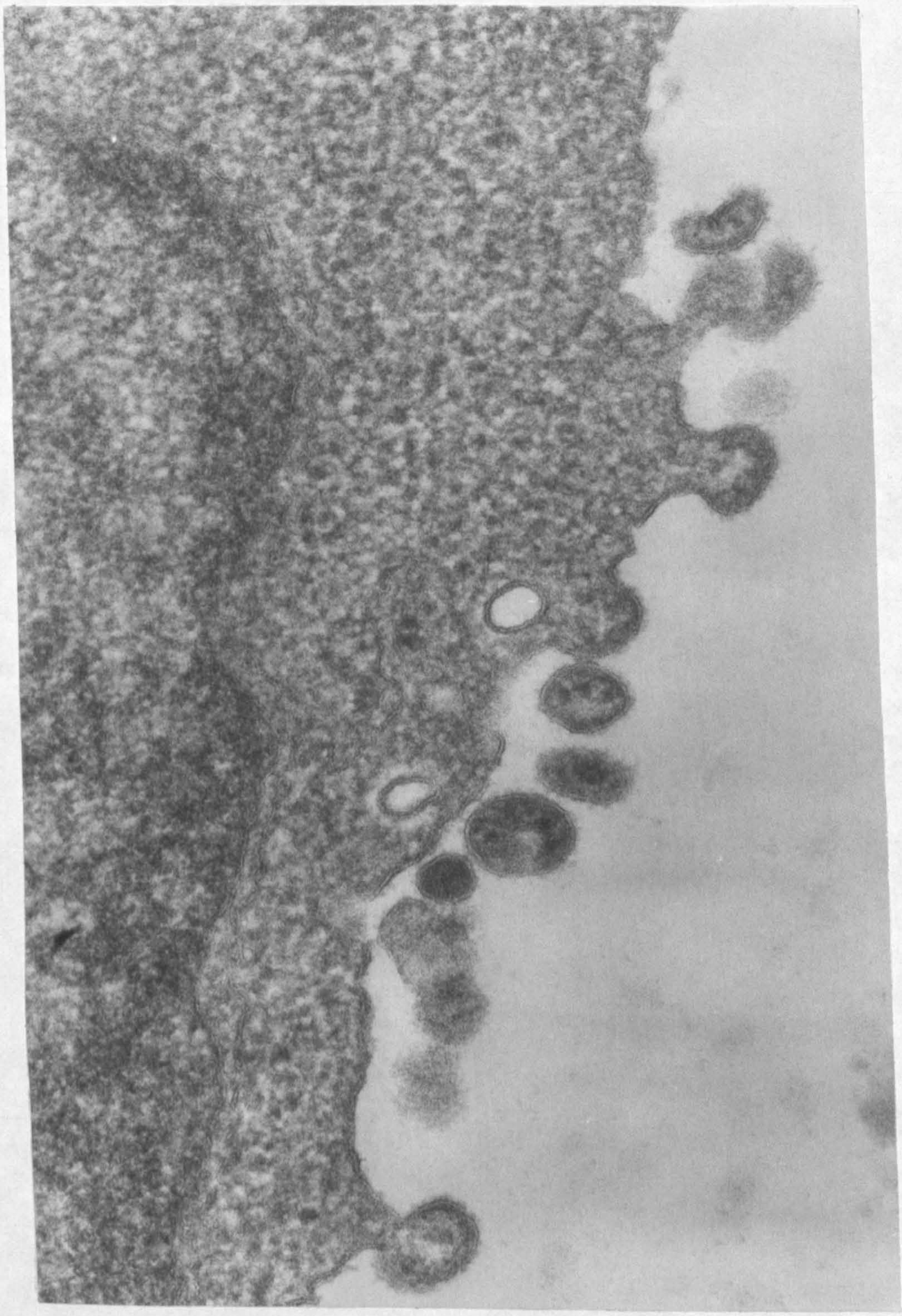


Fig.33. M148 virus infected Vero cell (day 5 p.i.) showing distinct unit-membrane envelopes especially at sites of particle budding. Surface projections on budding particles are also visible. The cell also shows a high concentration of dense granules within the cytoplasm beneath the altered plasma membrane. (x 115,000)

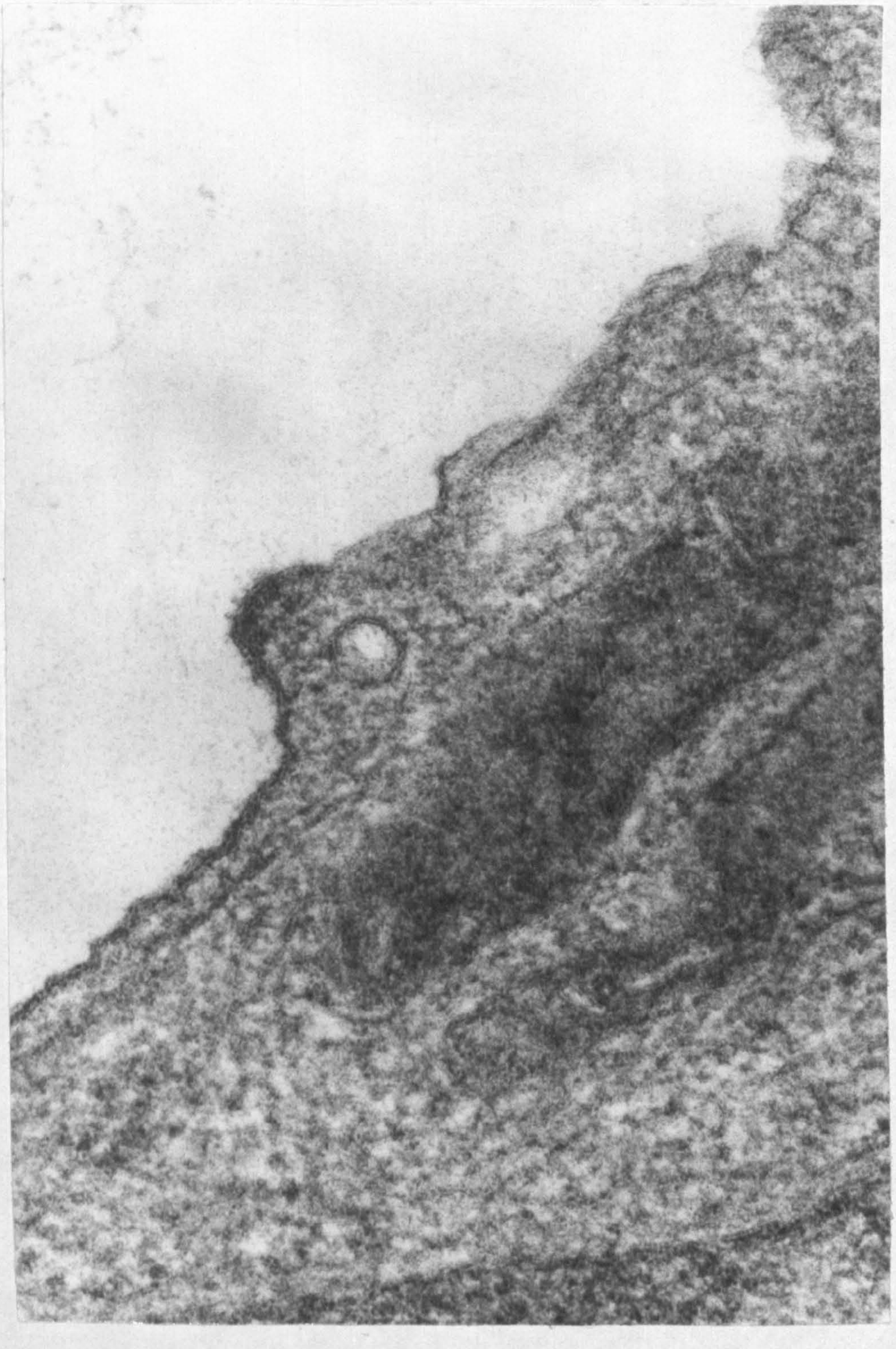


Fig.34. M150 virus infected Vero cell (day 2 p.i.) showing a distinct thickening of the plasma membrane along the whole length of the cell with one particle budding. Surface projections on the budding particle are also visible. (x 150,000)

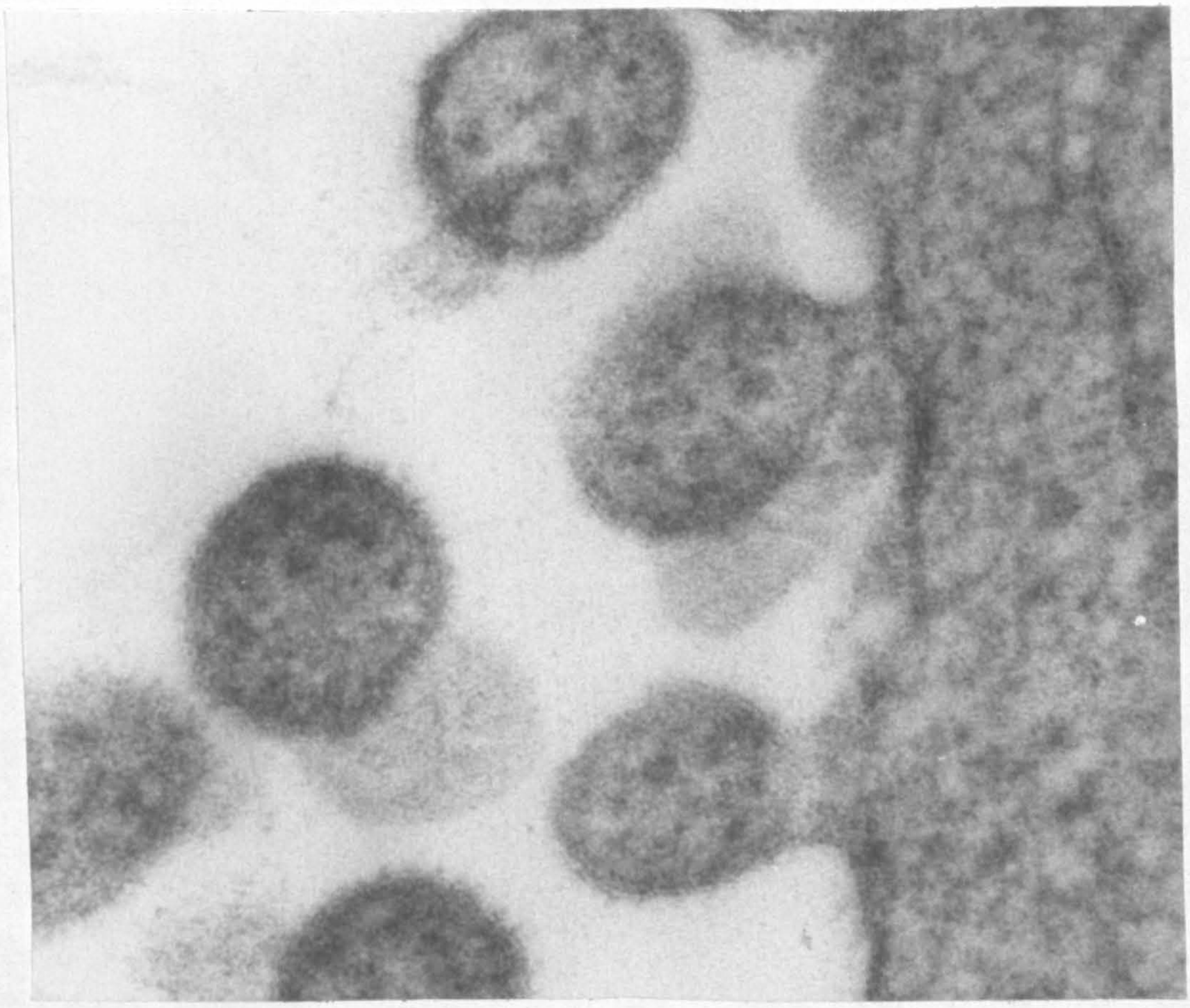


Fig.35. M150 virus particles in extracellular space at 6 days p.i. from Vero cells. (50,000)

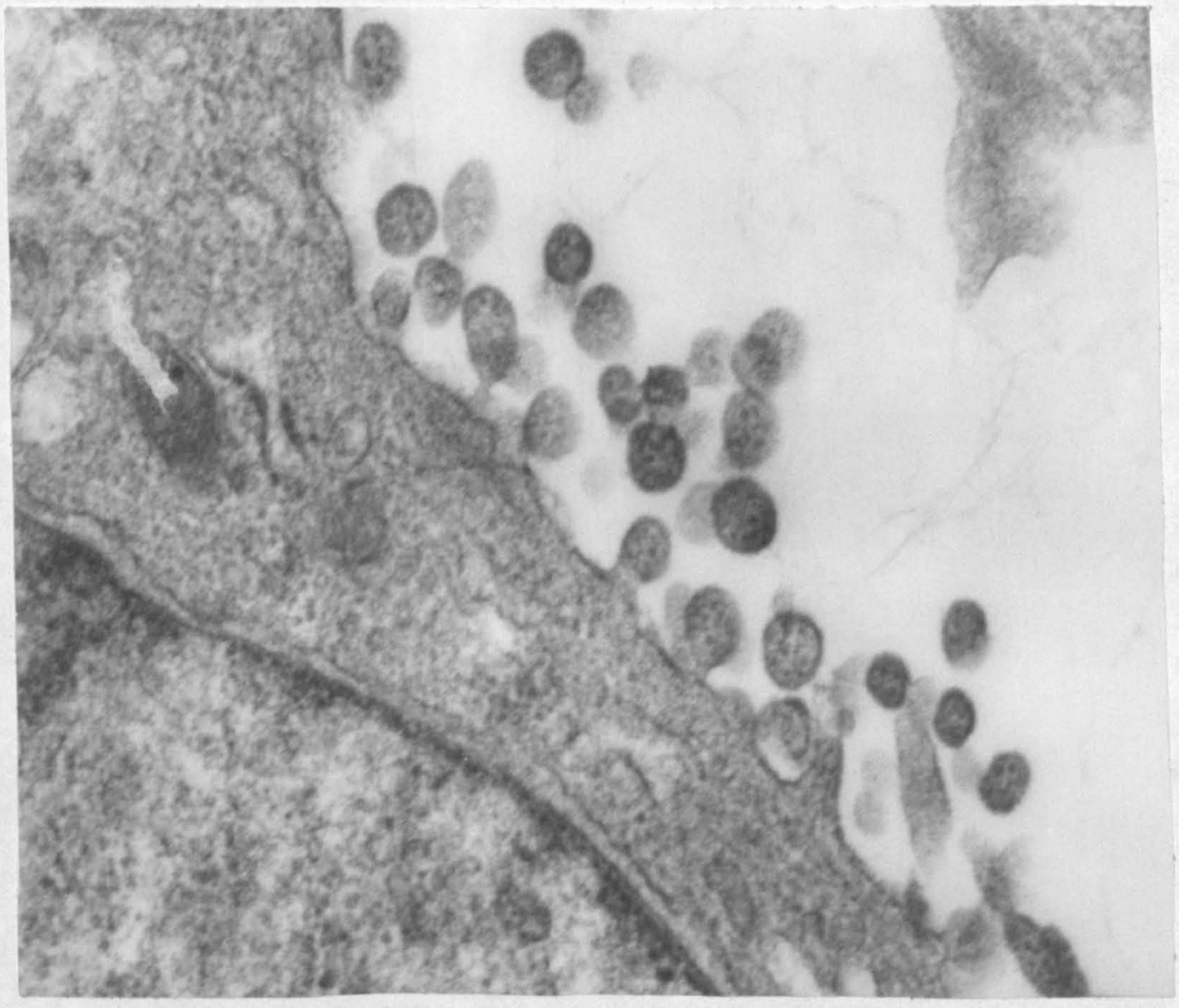


Fig.36. M150 virus particles at final stages of budding from Vero cells (day 5 p.i.). Particles demonstrate surface projections on unit-membrane envelopes. (x 200,000)

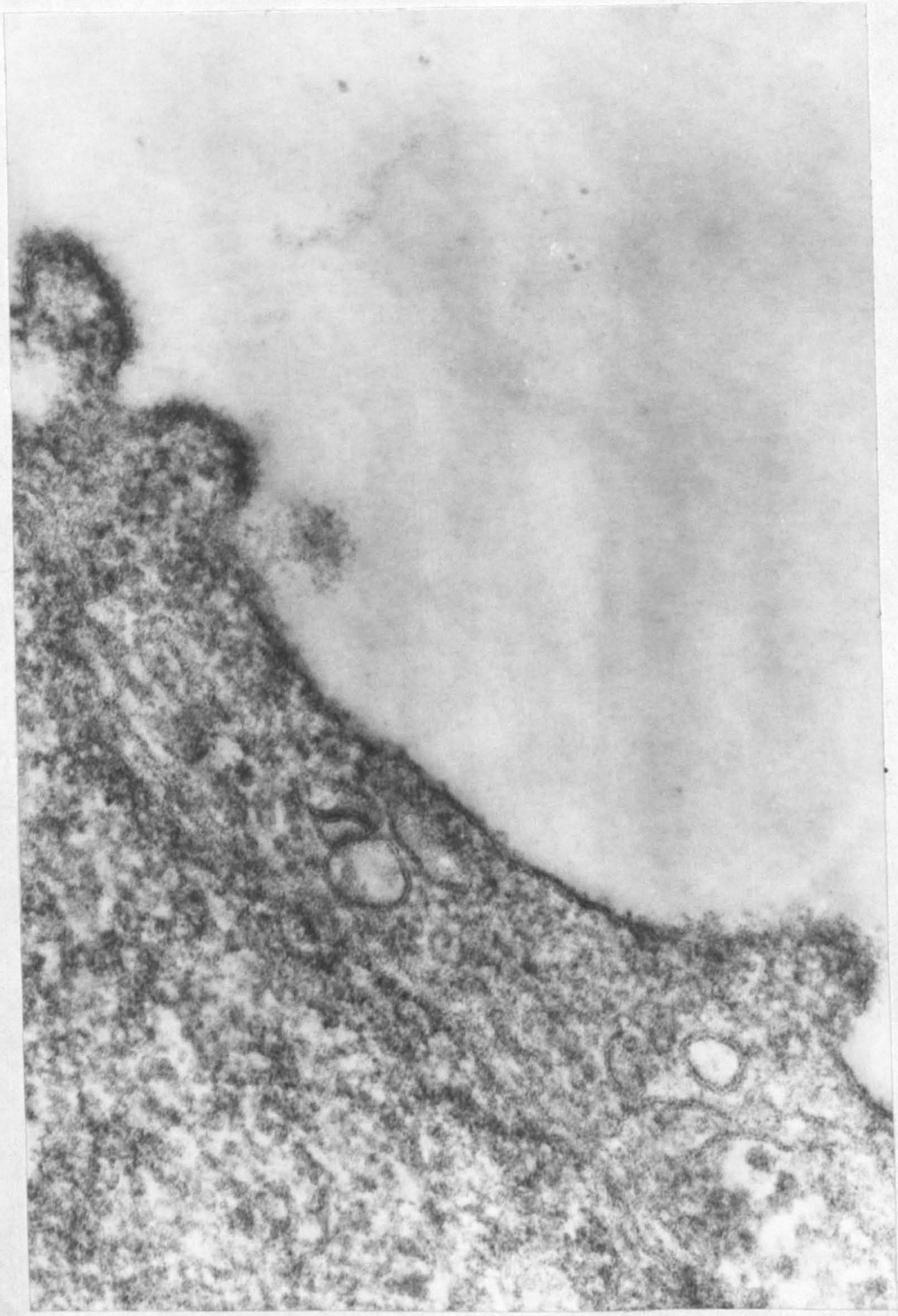


Fig.37. M152 virus infected Vero cell (day 3 p.i.) demonstrating characteristic arenavirus distinct unit-membrane envelope at site of particle budding. Even at early stages of budding, surface projections are visible. Cytoplasmic dense granules also in evidence below thickened cellular membrane. (x 150,000)

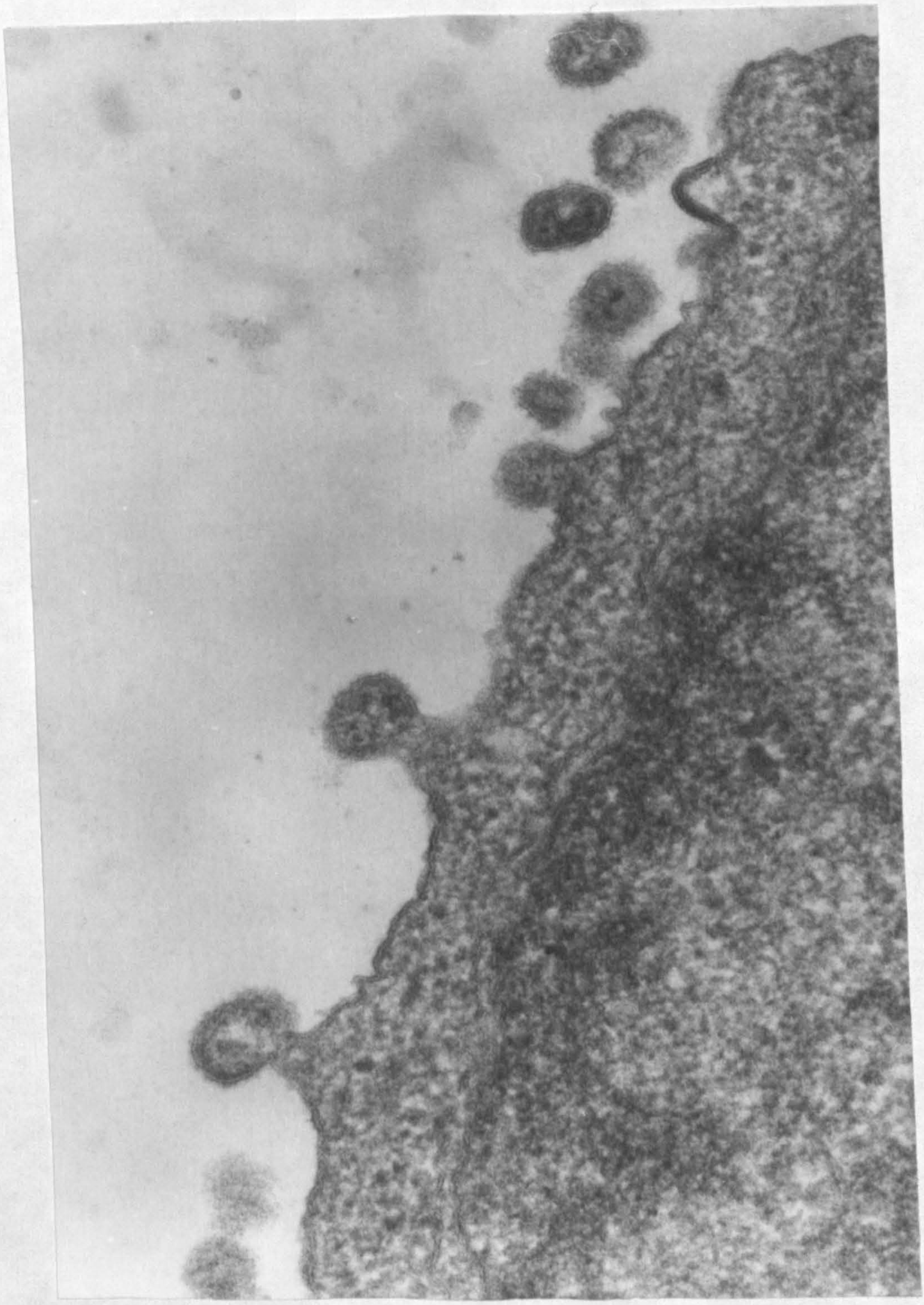


Fig.38. M152 virus infected Vero cell (day 5 p.i.) showing final stages of typical arenavirus budding from cell surface. Each particle containing 20-25 nm dense granules. (x 115,000).

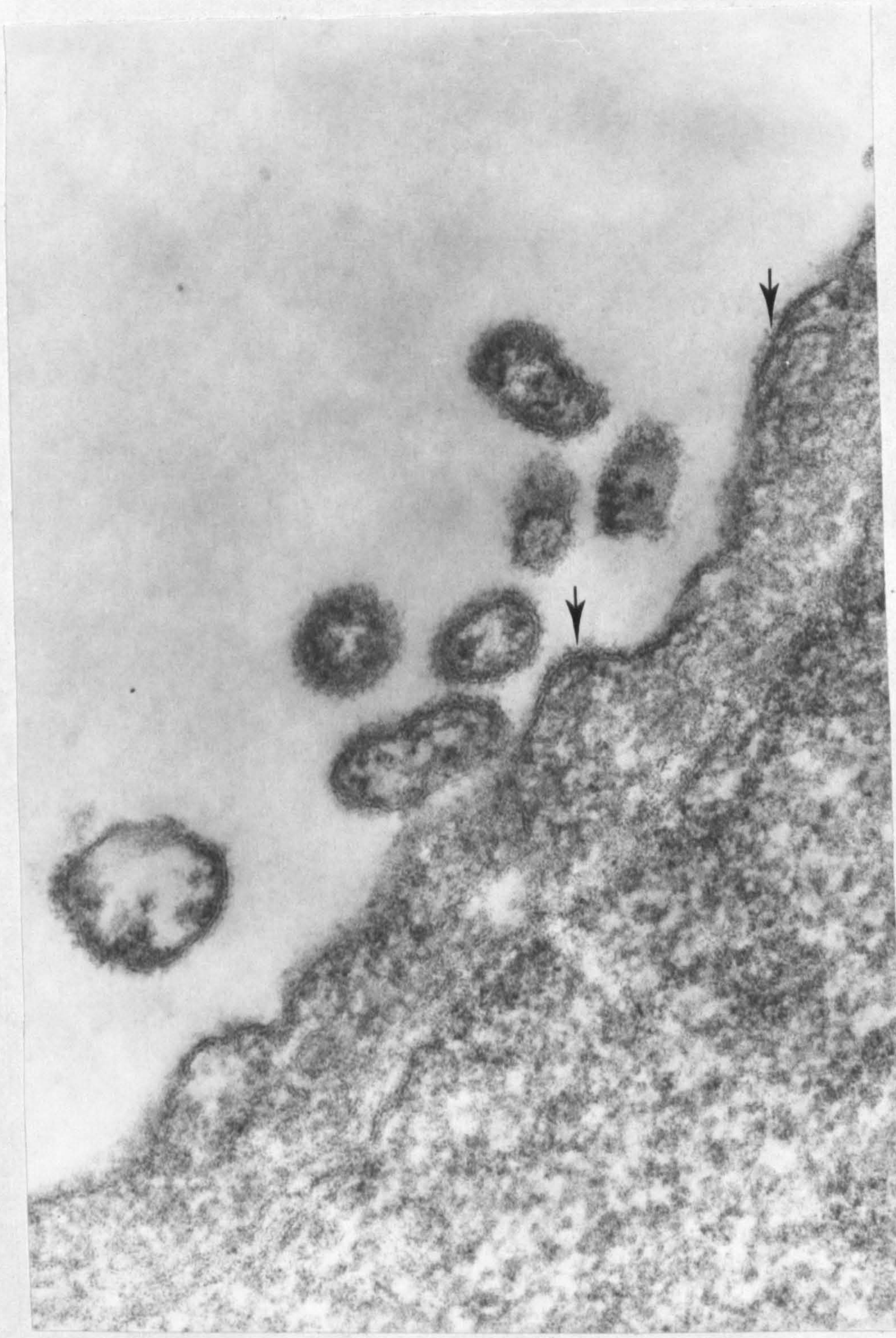


Fig.39. M152 virus infected cells (day 6 p.i.) demonstrating virus particles in extracellular space. A varying number of dense particles, double membrane and surface projections are visible. Budding is also in progress at other sites on the cell surface (arrows), (x 150,000)

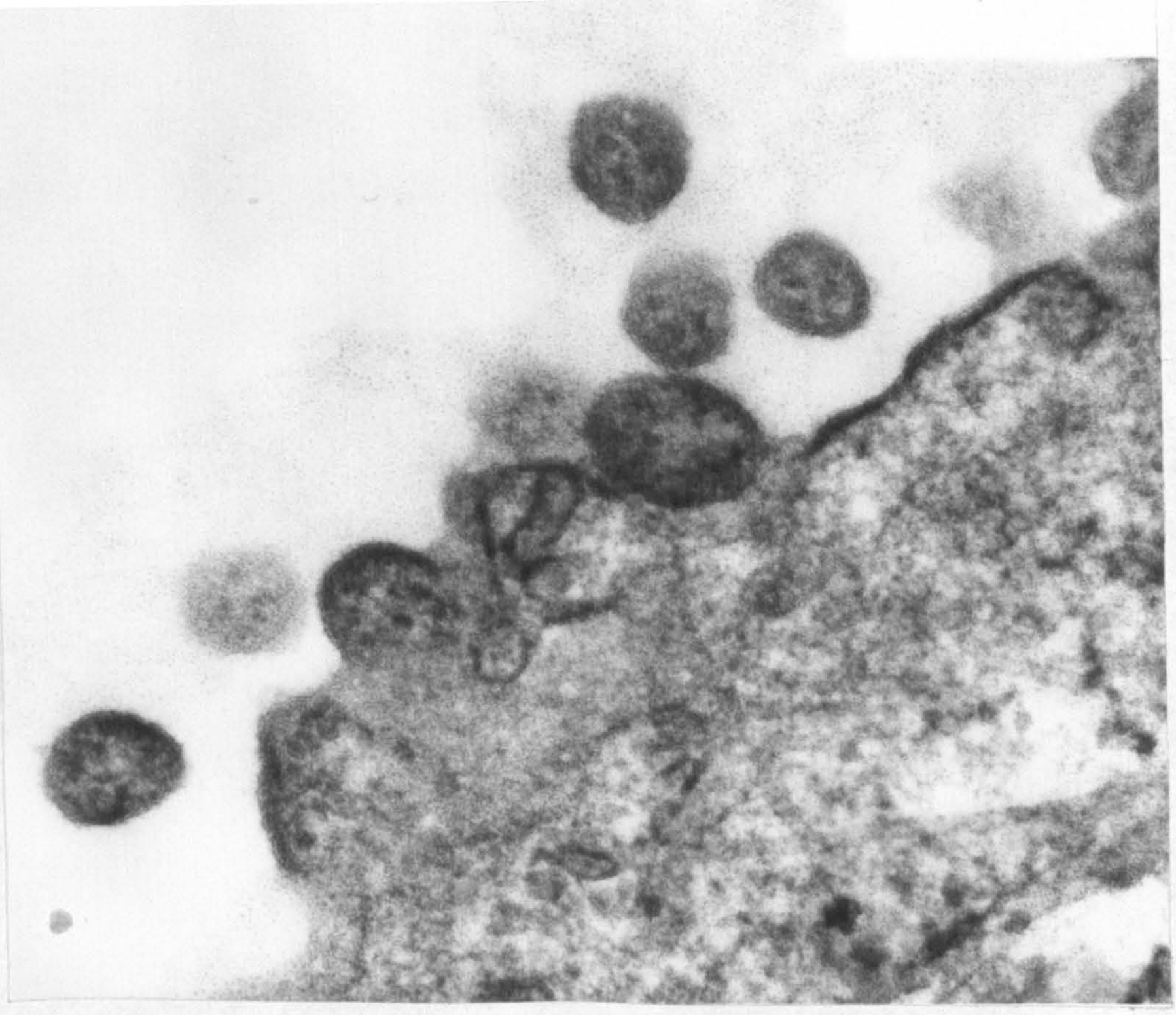


Fig.40. Z478 virus infected Vero cells (day 5 p.i.) showing various stages of typical arenavirus development. (x 150,000).

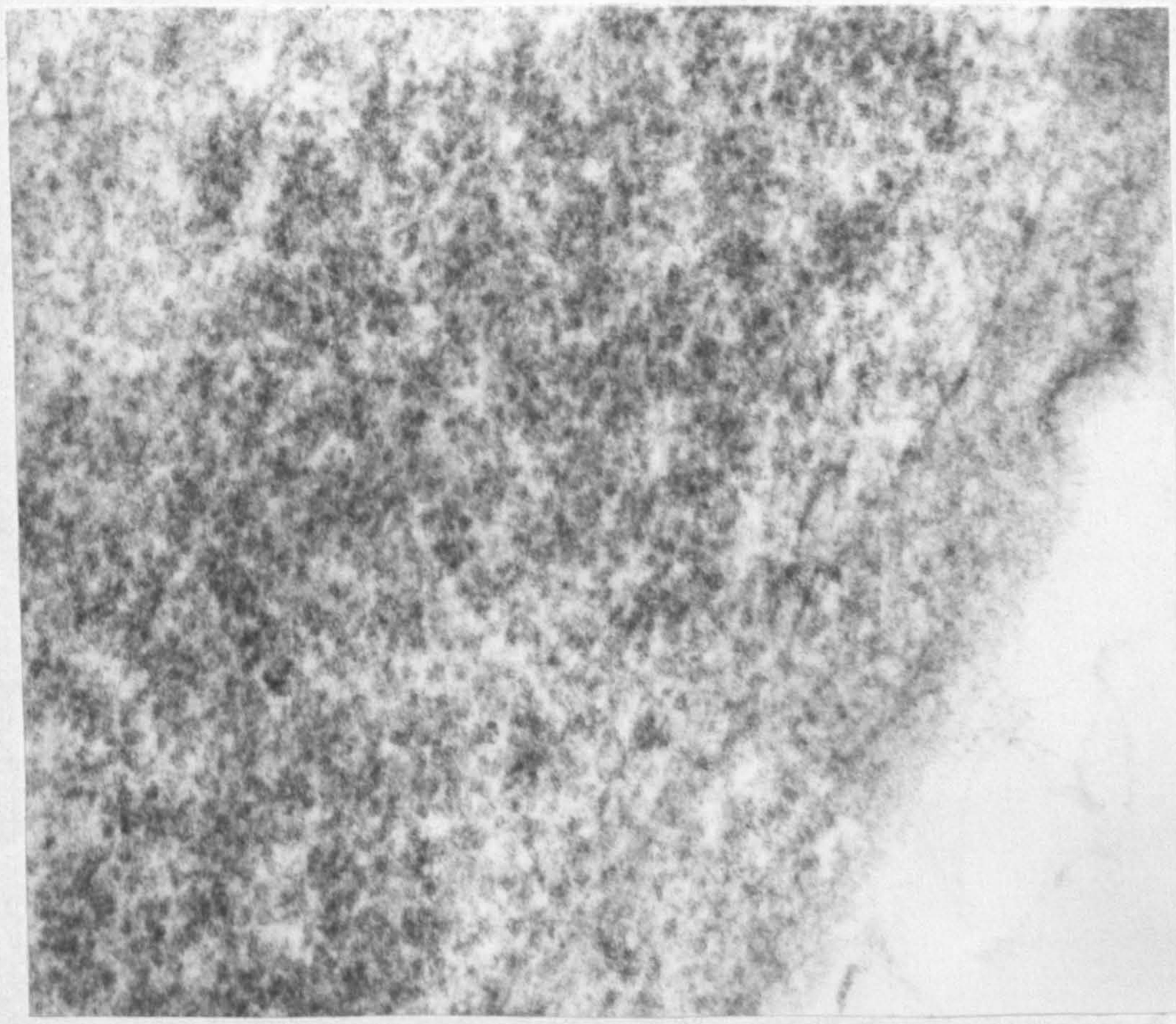


Fig.41. Z478 virus inclusion at a stage of infection preceding cytopathic changes. An increase in packing occurred late in infection, making inclusions appear very dense. (x 80,000)

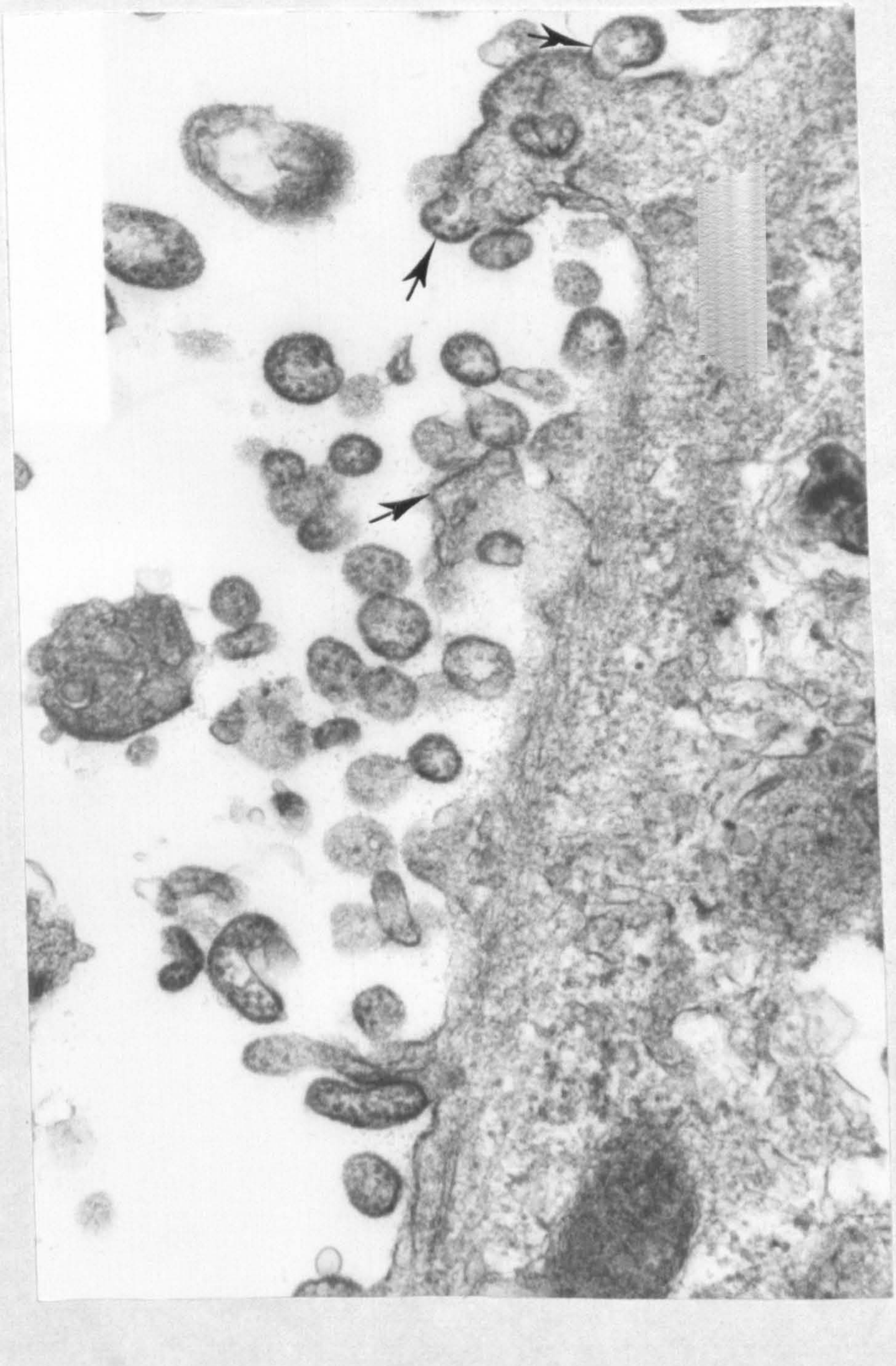


Fig.42. Z478 virus particles budding from plasma membrane over an extensive portion of Vero cell surface (arrows)(day 4 p.i.). The entire surface of some cells was involved in virus production. (x 52,000)

cytopathic changes that include rarefaction, condensation and lysis as stages in the common terminal necrotic pathway.

3.3. PHYSICOCHEMICAL STUDIES

The work outlined in this section was aimed at determining the physicochemical characteristics of Mopeia virus compared with LGA 391 and relating the findings to the arenavirus family. It was also aimed at using the data to comment on various clinical and work practices.

3.3.1. Thermal inactivation

The rate of inactivation of Lassa and Mopeia viruses was measured in the temperature range 28-60°C. Survival curves were constructed by plotting the reduction of infectivity at different times (Figs. 43-47). When a virus is inactivated according to a first order reaction the rate constant is given by the formula:

$$K' = (\ln V_t/V_o)t$$

where V_o is the original concentration of infectious virus and V_t the concentration of surviving virus at a given time.

An exponential decrease in infectivity of all viruses, without an initial shoulder, could be observed between 28-60°C. Thus the inactivation proceeded as a first-order reaction implying that the virus preparations were homogeneous. At the higher temperatures (50°C and 60°C) a two component curve was observed, a finding which has been occasionally recognised with some other RNA viruses (Ginoza, W., 1968).

Reference to Table 11 indicates the rate of their inactivation, the time taken to achieve a 50% reduction in infectivity (half-life t) and total time taken to reduce infectivity to undetectable limits.

The different velocity constants were plotted against temperature according to an Arrhenius diagram. As shown in Fig. 48, the plot is composed of two straight lines which intercept at about 45°C. The slope of the curves, which are related to the enthalpy change, were not

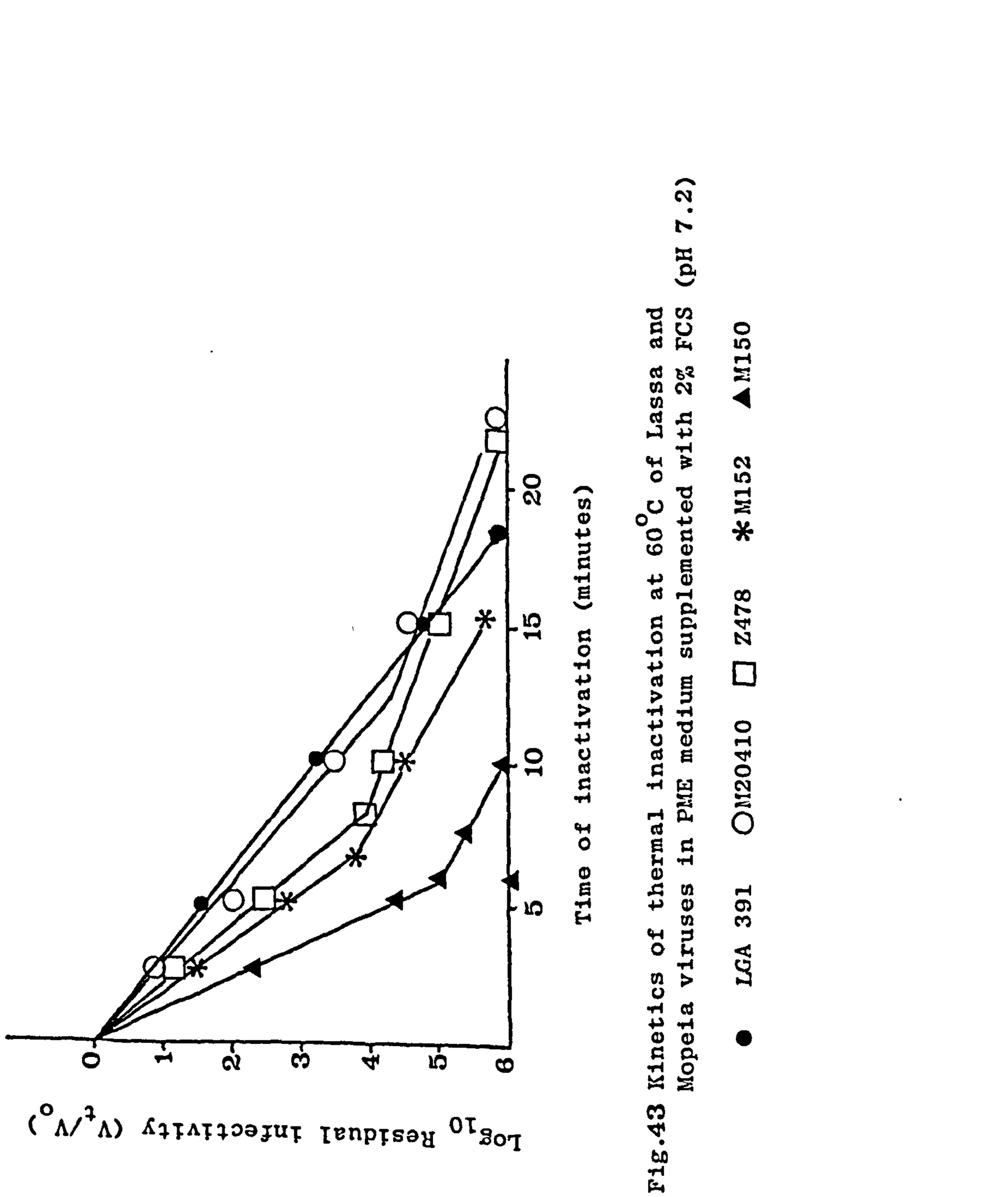


Fig.43 Kinetics of thermal inactivation at 60°C of Lassa and Mopeia viruses in PME medium supplemented with 2% FCS (pH 7.2)

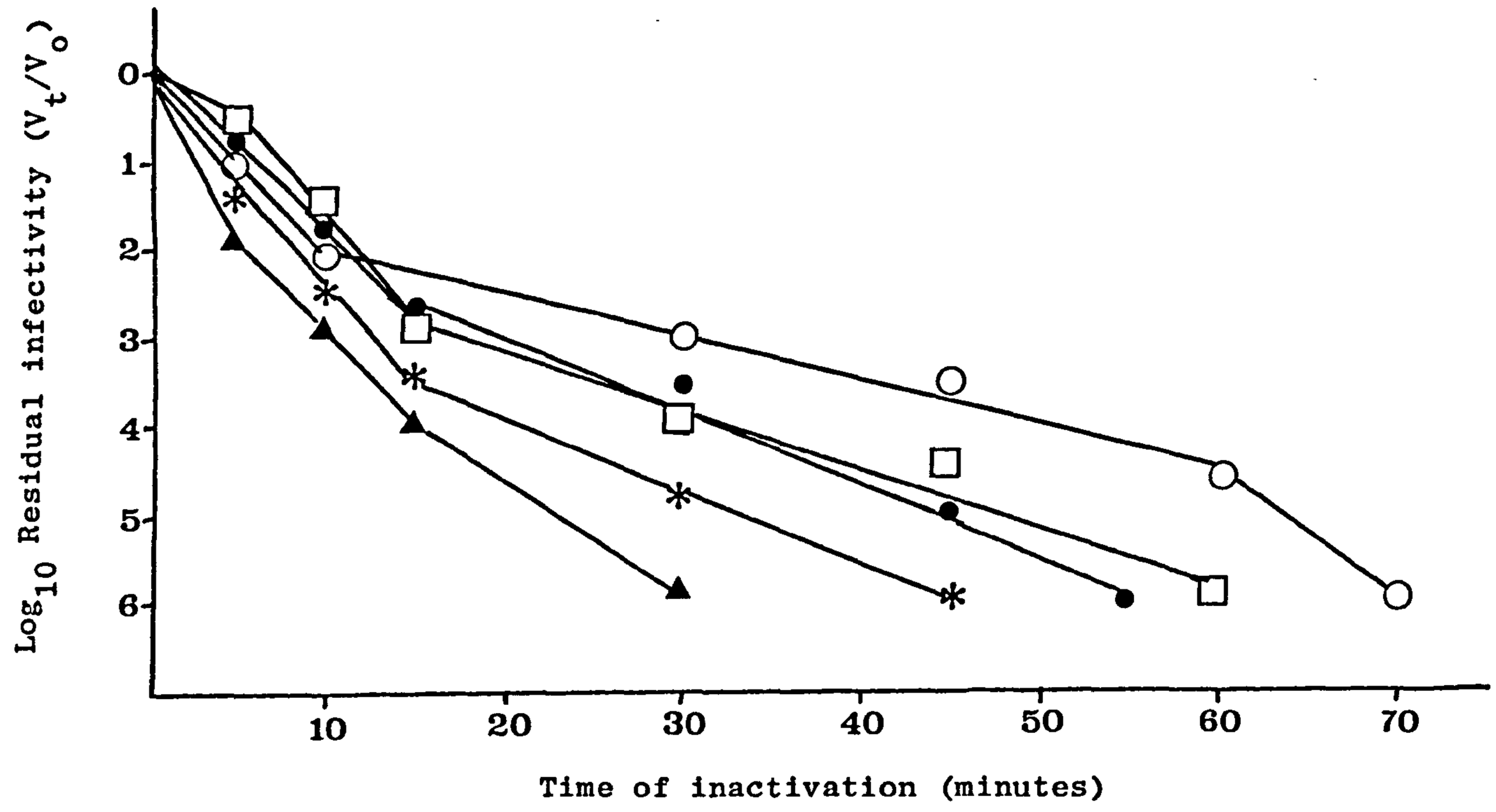


Fig.44 Kinetics of thermal inactivation at 56°C of Lassa and Mopeia viruses in PME medium supplemented with 2% FCS (pH 7.2)

● LGA 391 ○M20410 □ Z478 ✱M152 ▲M150

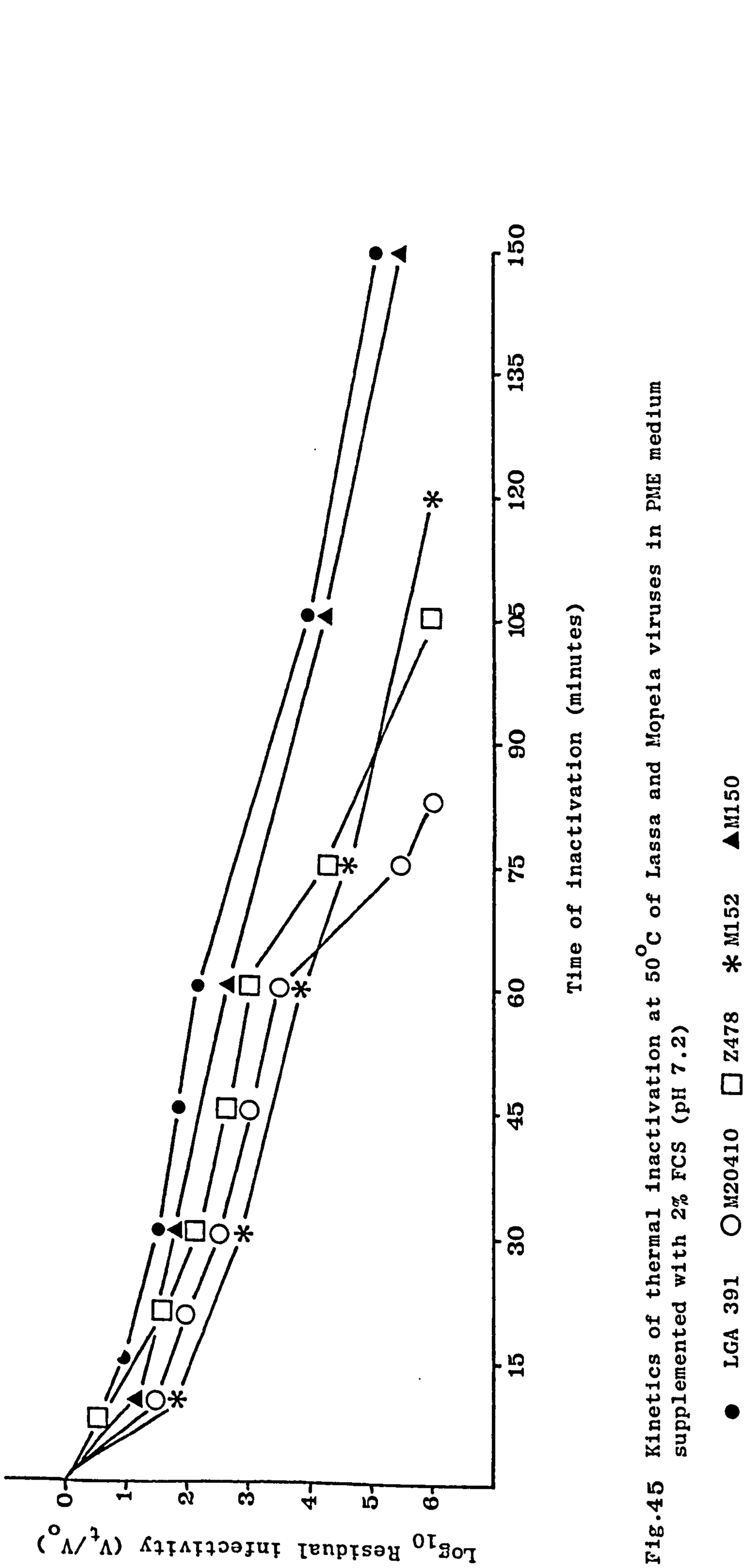


Fig.45 Kinetics of thermal inactivation at 50°C of Lassa and Mopeia viruses in PME medium supplemented with 2% FCS (pH 7.2)

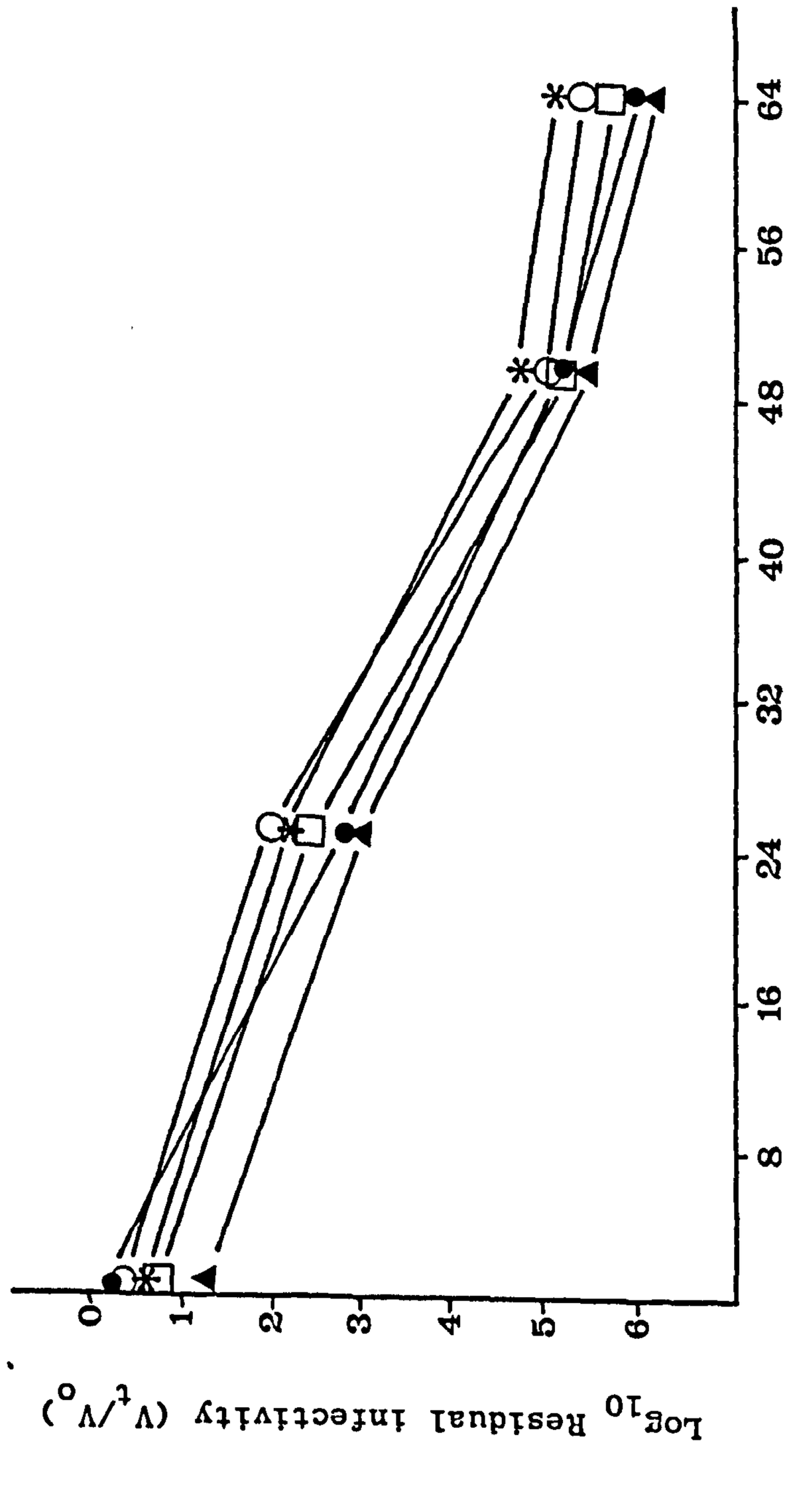


Fig.46 Kinetics of thermal inactivation at 37°C of Lassa and Mopeia viruses in PME medium supplemented with 2% FCS (pH 7.2)

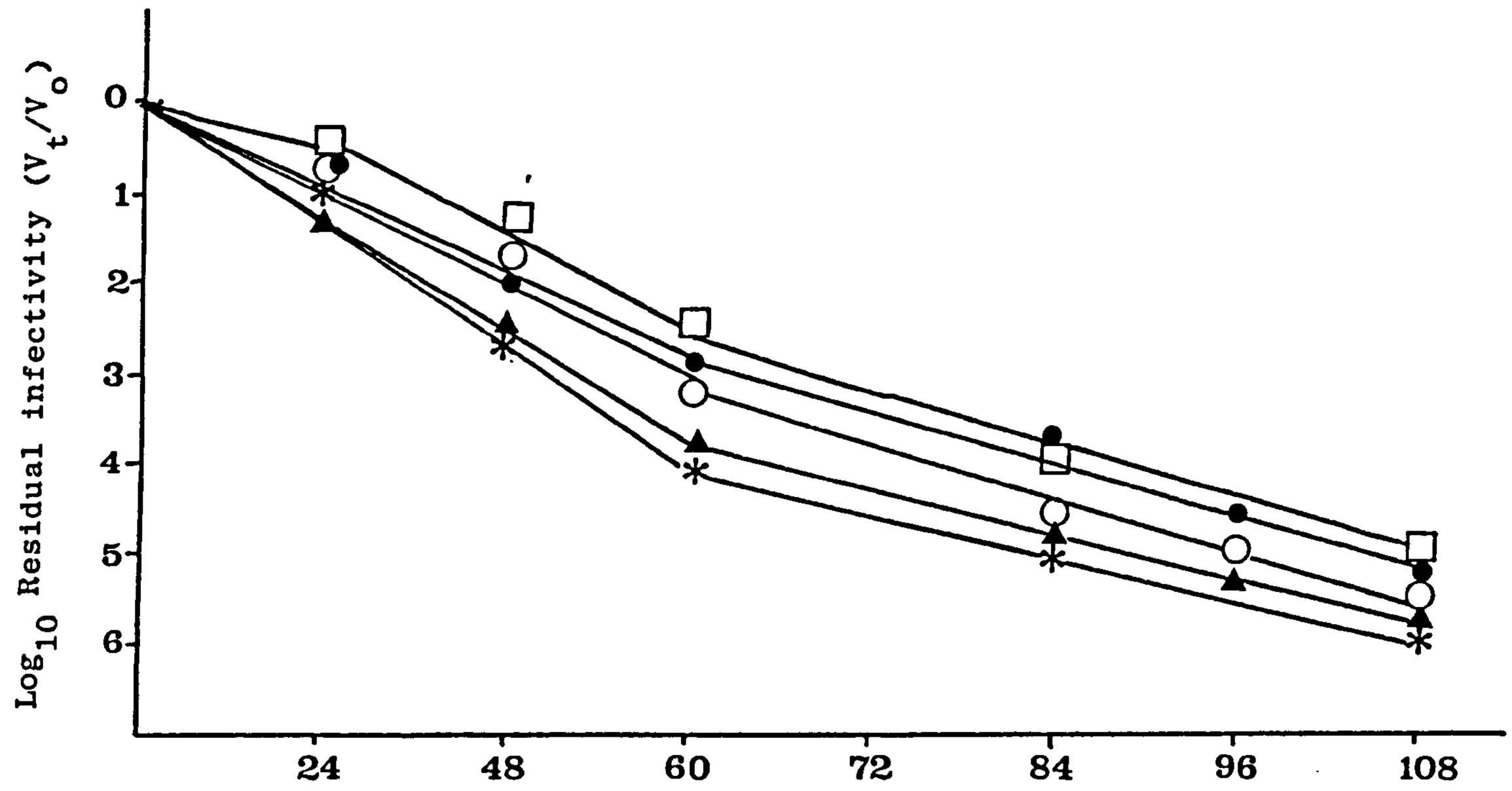


Fig.47 Kinetics of thermal inactivation at RT (28°C) of Lassa and Mopeia viruses in PME medium supplemented with 2% FCS (pH 7.2)

● LGA 391 ○M20410 □Z478 * M152 ▲M150

TABLE 11. EFFECT OF VARIOUS TEMPERATURES ON LASSA AND MOPEIA VIRUSE

Virus	Temp °C	Half-life t'	Total inactivation Time	Velocity constant K' (min^{-1})
LGA391	60	2.0 mins	18 mins	0.84
	56	3.0 "	55 "	0.63
	50	7.5 "	150 +* mins	0.076
	37	1 hour	64 + hours	0.004
	28	18 hours	108 + "	0.002
M152	60	0.6 mins	15 mins	1.03
	56	1.0 "	45 "	0.53
	50	2.5 "	120 + mins	0.23
	37	1 hour	64 + hours	0.004
	28	12 hours	108 "	0.002
M150	60	0.5 mins	10 mins	1.68
	56	1.0 "	30 "	0.76
	50	2.5 "	150 + mins	0.07
	37	1 hour	64 hours	0.004
	28	12 hours	108 "	0.002
M20410	60	2.0 mins	22 mins	0.83
	56	2.5 "	80 "	0.57
	50	3.5 "	150 + mins	0.19
	37	1 hour	64 + hours	0.004
	28	24 hours	108 + "	0.002
Z478	60	0.6 mins	22 mins	0.92
	56	2.5 "	60 "	0.46
	50	4.0 "	105 "	0.13
	37	1 hour	64 + hours	0.004
	28	24 hours	108 + "	0.002

* + = experiment terminated before complete inactivation

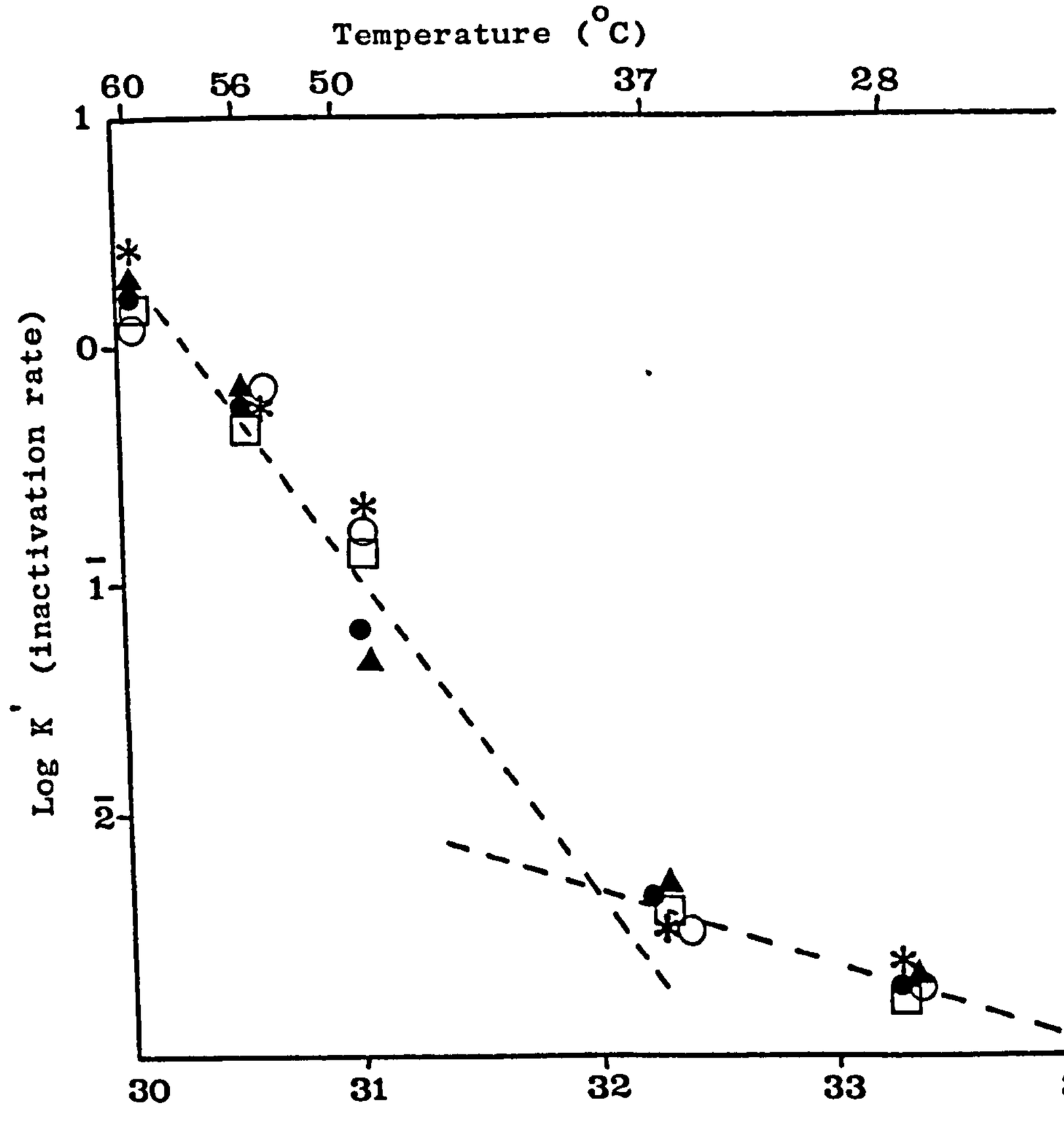


Fig.48 Arrhenius plots for thermal inactivation of Lassa and Mopeia viruses. Logarithms of rate constant were plotted against reciprocal of absolute temperature. The K' values were derived from fast components of Figs.43-47 by the formula:

$$K = \frac{V_o}{V_t} \cdot \frac{2.3}{t}$$

where V_o = original virus concentration

V_r = virus concentration at time t

t = exposure time (minutes)

$\frac{1}{T}$ = reciprocal of absolute temperature

Dotted line represents the trend for each virus.

● LGA 391 ○ M20410 □ Z478 ✱ M152 ▲ M150

significantly different when the data for each virus was plotted. From each component it was possible to obtain the thermodynamic constants for the overall process of inactivation which they described (Table 12). These were the energy of inactivation (ΔH) and the entropy of activation (ΔS) and were calculated from inactivation velocity constants according to Ginoza (1968).

TABLE 12. THERMODYNAMIC PARAMETERS FOR THE INACTIVATION OF LASSA AND MOPEIA VIRUSES

Virus	Temp. °C	ΔG (Kcal/mol)	ΔH (Kcal/mol)	ΔS (cal.mol/°C)
LFGA391	50 - 60	11.8 - 17.8	100	265
	28 - 45	24.3 - 25.5	22.10	-9.3
M152	50 - 60	11.1 - 15.0	64.02	155
	28 - 45	24.9 - 25.1	20.78	-13.8
M150	50 - 60	9.9 - 18.1	131.78	360
	28 - 45	24.5 - 25.3	22.19	-9.2
M20410	50 - 60	11.9 - 15.4	59.24	139
	28 - 45	24.8 - 25.4	19.95	-16.8
Z478	50 - 60	11.5 - 16.1	76.45	191
	28 - 45	24.8 - 25.7	22.44	-9.4

The ΔH and ΔS values are consistent with those reported for RNA containing viruses (Ginoza, 1968) and suggest the existence of two different inactivation reactions at temperatures below and above 40°-45°C. The results also demonstrate a consistency in reaction by Lassa and Mopeia viruses to thermal inactivation.

3.3.2. Effect of ultraviolet light

Ultraviolet light reduced the infectivity titre from 10^7 to 10^1 $TCID_{50}$/ml in 15-20 seconds. This reduced further after 30 seconds to a

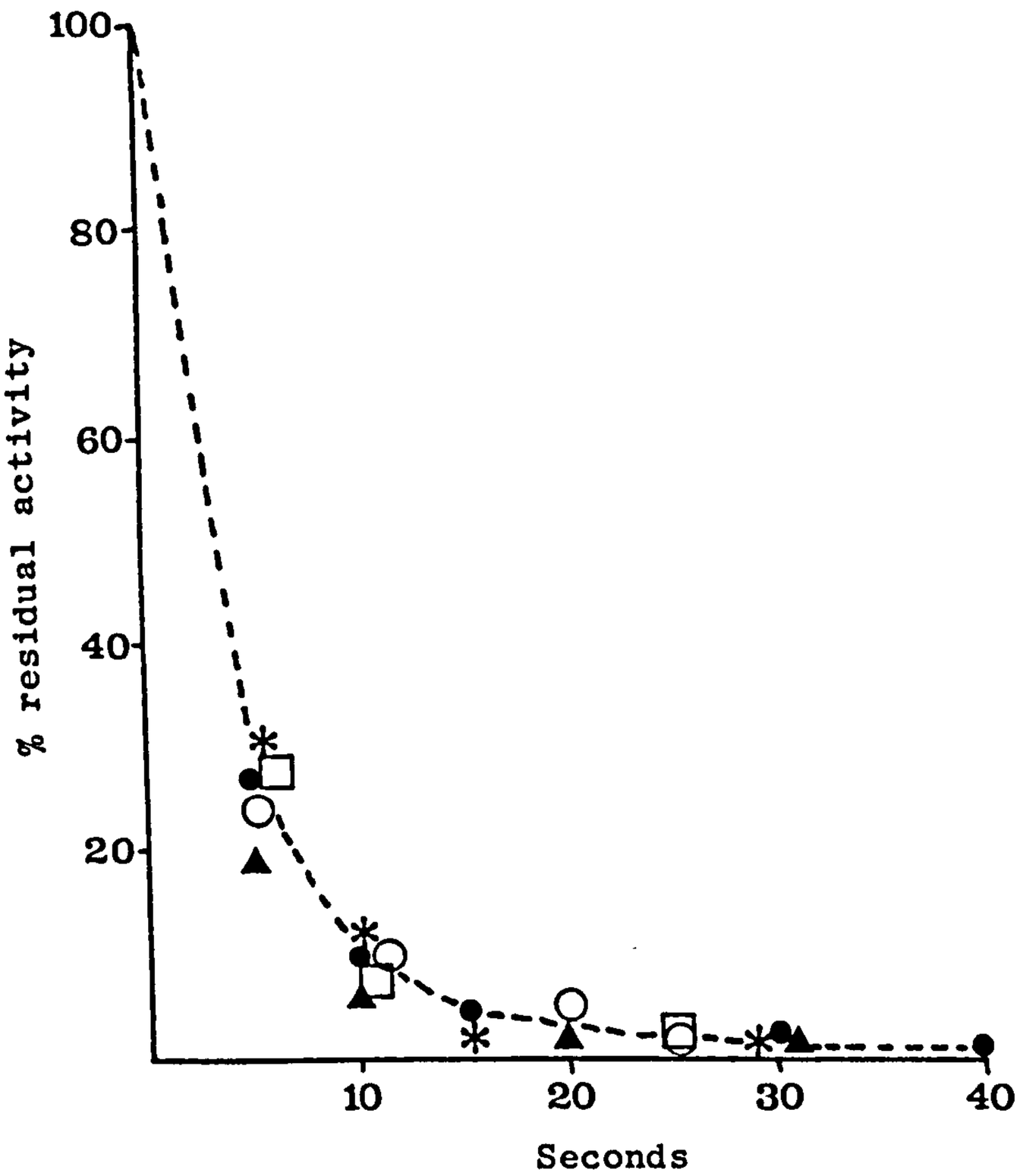


Fig.49 Inactivation rate of Lassa and Mopeia viruses irradiated with UV light. Each point represents the mean of 5 separate experiments.

● LGA391 ○ M20410 □ Z478

✱ M152 ▲ M150

level where only the undiluted tissue culture fluid (PMB) was positive. No detectable virus was made beyond 40 seconds. All arenaviruses reacted the same way as demonstrated in Fig.49.

3.3.3. pH sensitivity

Infectivity reductions at various pH levels for Lassa and Mopeia viruses are depicted in Fig.50. Of the Mopeia strains, M152 remains stable between pH 6.6 - pH 8.0. Lassa GA 391 has a pH stability range between pH 6-8.5. Less than one $\log_{10}$ reduction in titre occurred at pH 8.0 and above for LF GA 391 and M152. A larger reduction in infectivity (10^1-10^2 $\log_{10}$) was observed with M20410, M150 and Z478 at pH 8.0 and above.

In all cases complete inactivation was observed at pH 5.0 and below .

Table 51 shows the effects at different pH levels on suspensions of Lassa and Mopeia viruses maintained for 6 hours and 18 hours at +4°C. The decrease of infectivity was not significant between pH 6.5 and 9.5 after 6 hours at +4°C or between pH 6.5 and 9.0 after 18 hours. Complete inactivation was achieved at below pH 5.5. This applied to all the viruses studied.

TABLE 13. EFFECT OF pH ON LGA 391 AND MOPEIA VIRUSES

pH	Time of incubation (hours)	% Inactivation				
		LF	M152	M150	M20410	Z478
4.5	6	99.9	99.9	99.9	99.9	99.9
5.5	"	"	"	"	"	"
6.5	"	0	0	0	0	0
7.5	"	0	0	0	0	0
8.5	"	5	6	10	6	7
9.5	"	8	7	8	9	10
4.0	18	99.9	99.9	99.9	99.9	99.9
4.5	"	"	"	"	"	"
5.5	"	"	"	"	"	"
6.5	"	10	10	17	15	20
7.5	"	21	12	14	15	24
8.5	"	18	12	15	14	18
9.5	"	35	40	42	38	43

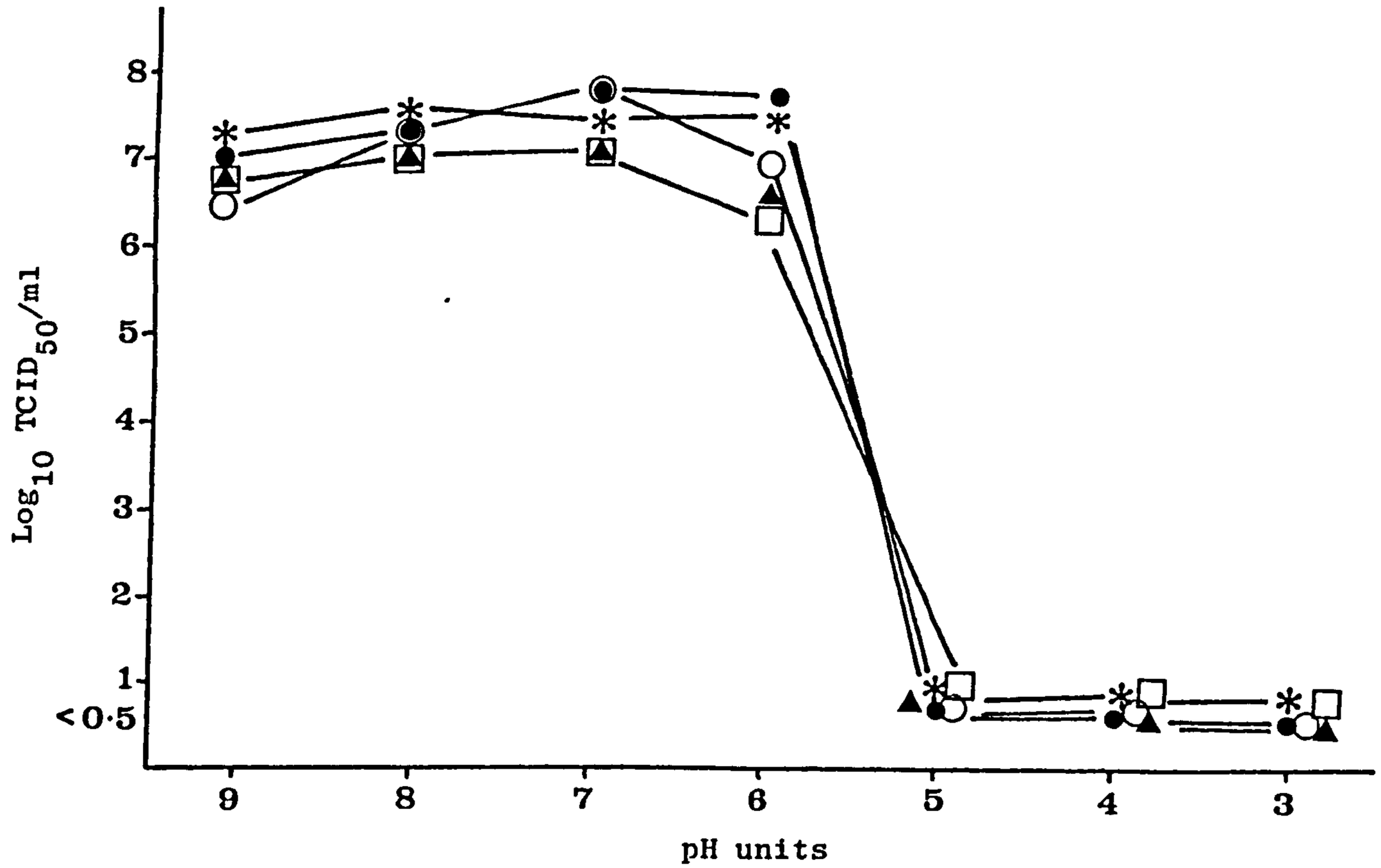


Fig.50 Effect of pH on the infectivity of Lassa and Mopeia viruses.

● LGA391 ○M20410 □ Z478 ✱ M152 ▲M150

3.3.4. Effects of solvents

Ether and chloroform reduced the infectivity titre of Mopeia and LGA 391 viruses. These viruses were therefore considered to be ether sensitive but not completely inactivated under the conditions of the test. After acetone and alcohol treatment both Lassa and Mopeia virus was recovered at the end of the test period. No recovery of any of the viruses resulted from sodium desoxycholate treatment (Table 14).

TABLE 14. EFFECT OF ETHER, CHLOROFORM, ALCOHOL, ACETONE AND SODIUM DESOXYCHOLATE

	Titre of virus*					
Virus	Ether	Chloroform	Alcohol	Acetone	Sodium desoxycholate	Control
LGA391	1.5	1.0	5.0	2.8	<0.5	7.0
M20410	<0.5	<0.5	5.5	3.3	"	6.5
M150	<0.5	<0.5	5.6	2.4	"	6.8
M152	1.0	<0.5	6.1	3.4	"	7.2
Z478	<0.5	1.0	5.0	2.4	"	5.8

*Expressed as $TCID_{50}$/ml

3.3.5. Effect of formaldehyde and glutaraldehyde

The effect of formaldehyde and glutaraldehyde on LGA 391 and Mopeia viruses was observed to be similar irrespective of the temperature being used (4°C, 28°C, 37°C). The concentrations of main interest were 10%, 3-4%, and 0.02% for the following reasons. The 10% concentration has long been used as a requirement for decontamination under high containment (CAMR Code of Practice); 3-4% are the levels used for EM preparation and 0.02% for the inactivation of material used in immunisation protocols.

The results of the LGA 391 and Mopeia virus inactivation studies with formaldehyde and glutaraldehyde were very similar irrespective of the concentration and the temperature conditions used (Figs. 51 and 52). At

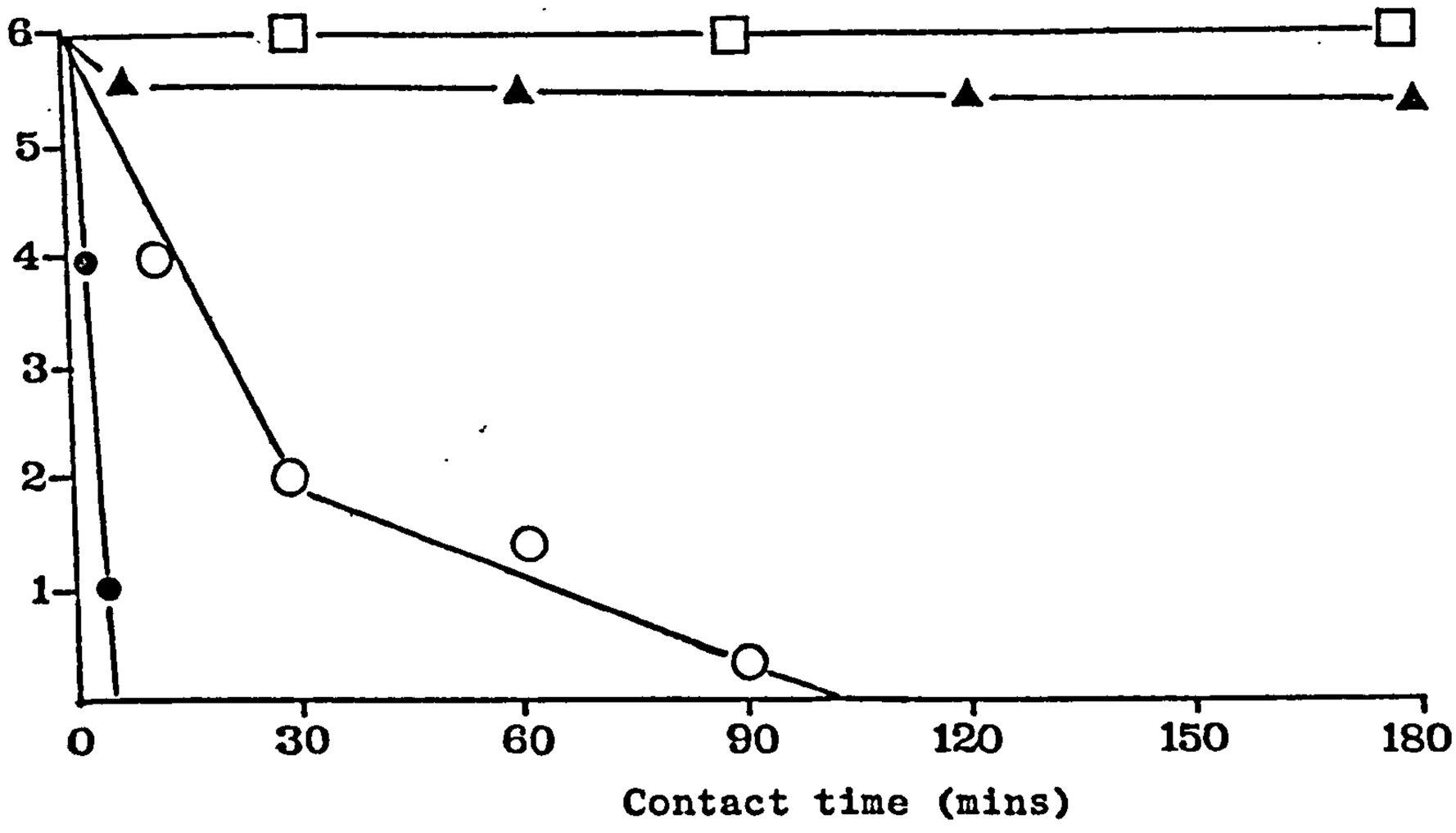


Fig.51 Effect of various concentrations of formaldehyde on LGA 391 at Room Temperature

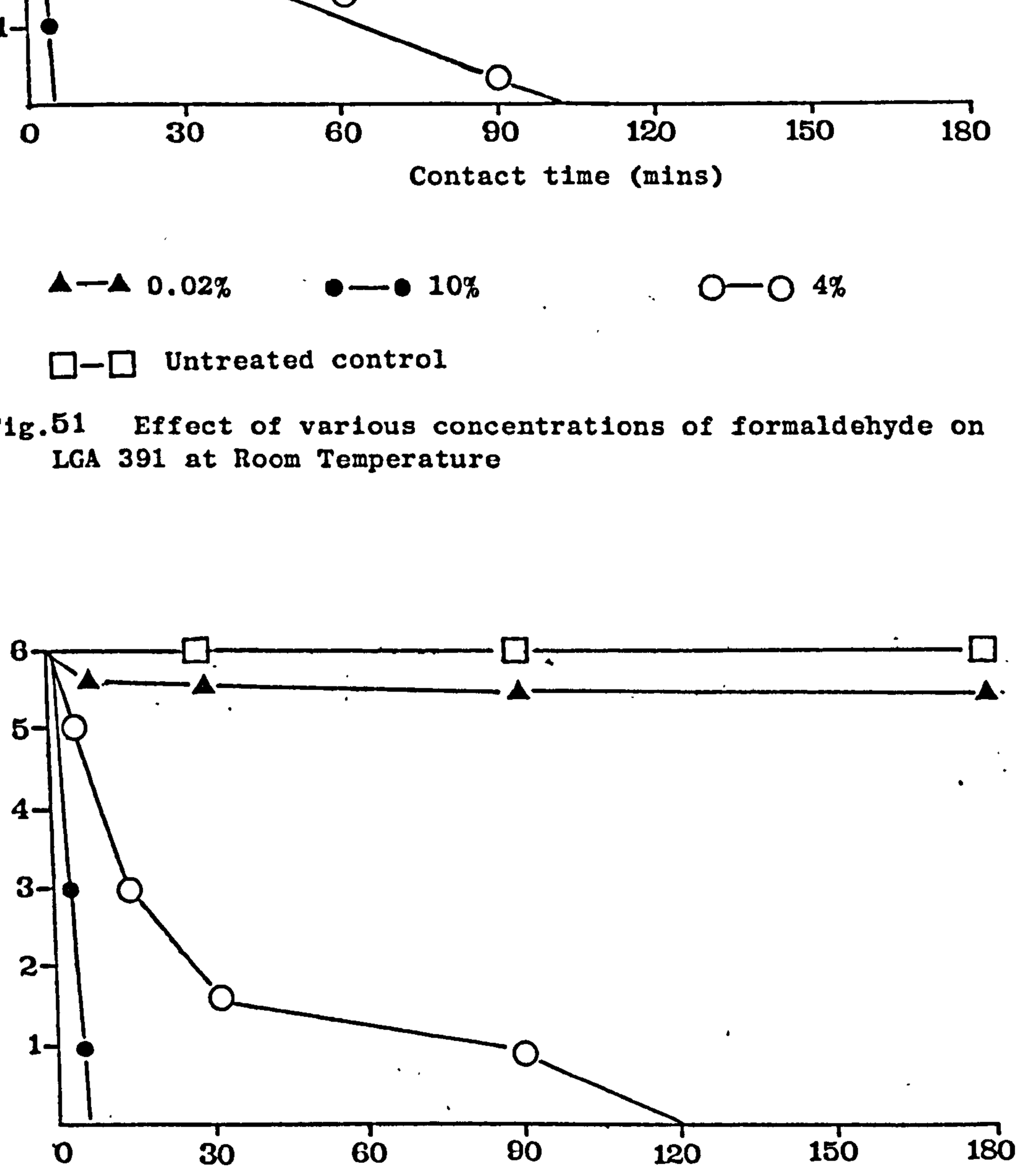


Fig.51 Effect of various concentrations of glutaraldehyde on LGA 391 at Room Temperature.

10% both formaldehyde and glutaraldehyde inactivated both LGA 391 and the Mopeia viruses (6 $\log_{10}TCID_{50}$) to undetectable levels within 5-10 minutes. Glutaraldehyde at a concentration of 3% inactivated 90% of Lassa and Mopeia virus populations within 15 minutes, the residual 10% being eliminated after 2 hours incubation. Formaldehyde at a concentration of 4% inactivated all viruses within 1½ hours to undetectable limits. The 0.02% levels of formaldehyde and glutaraldehyde did not inactivate the viruses until 5 days incubation had been completed but it was noted that the pH of the support medium changed from pH 6.5 to pH 3 which in itself has an inactivating effect. The EM studies carried out on the samples prepared in 3% glutaraldehyde were found to be suitable for structural studies even when the preparation was kept for 24 hours. Formaldehyde at a concentration of 4% appeared to structurally alter the virus particles upon EM study.

3.3.6. Effect of BPL concentrations

The aim of these studies was to establish the most appropriate working concentration for BPL to inactivate Lassa and Mopeia virus strains. The effect of different temperatures, time and type of media on BPL inactivation was considered.

In the case of Lassa infected sera (Table 15) a 0.1% solution of BPL used at 4°C reduced all virus titres to $10^{1.0}$-$10^{0.5}$ $TCID_{50}$/ml within 18 hours, and at 37°C the reduction was to between $10^{2.3}$-$10^{1.5}$ $TCID_{50}$/ml within 30 minutes. To obtain undetectable levels of virus it was found necessary to double the BPL concentration to 0.2%. In a human acute phase serum containing $10^{5.0}$ $TCID_{50}$/ml, 0.2% BPL reduced viral infectivity to undectable levels within 18 hours at 4°C and 30 minutes at 37°C. Similar data was observed using Guinea Pig and Monkey infected sera.

TABLE 15. INACTIVATION OF LGA 391 AND MOPEIA VIRUSES IN HUMAN SERA BY BPL

Conc of BPL%	Recovery of Virus (log_{10} $TCID_{50}$/ml)									
	a					b				
	LGA391	20410	478	150	152	LGA391	20410	478	150	152
0.3	-ve	-	-ve	-ve	-ve	-ve	-	-ve	-ve	ve
0.2	-ve	-	-ve	-ve	-ve	-ve	-	-ve	-ve	ve
0.1	<0.5	<0.5	1.0	<0.5	<0.5	2.3	1.2	1.5	2.0	1.8
0.05	2.0	2.6	3.1	2.9	3.5	4.8	3.6	2.8	4.2	3.8
0.025	5.2	4.8	6.5	6.0	6.8	6.5	5.8	6.8	6.0	6.8
Control	7.5	6.8	7.0	6.8	7.2	7.5	6.8	7.0	6.8	7.2

a. 4°C for 18 hours

b. 37°C for 30 minutes

In the case of tissue culture fluid containing between $10^{6.8}$-$10^{7.5}$ $TCID_{50}$/ml 0.1% BPL proved to be adequate in inactivating virus to undetectable levels (Table 16).

TABLE 16. INACTIVATION OF LGA 391 AND MOPEIA VIRUSES IN TISSUE CULTURE FLUID BY BPL

Conc of BPL %	Recovery of Virus (log_{10} $TCID_{50}$/ml)									
	a					b				
	LGA391	20410	478	150	152	LGA391	20410	478	150	152
0.3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	ve
0.2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	ve
0.1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	ve
0.05	6.8	5.0	6.8	6.0	6.5	5.5	4.5	6.0	5.8	6.5
0.025	7.5	6.8	7.0	6.5	7.2	7.0	6.7	7.0	6.8	7.2
Control	7.5	6.8	7.0	6.6	7.2	7.5	6.8	7.0	6.8	7.2

a. 4°C for 18 hours;
b. 37°C for 30 minutes

No Lassa or Mopeia viruses could be recovered from susceptible guinea pigs or mice after inoculation of BPL treated tissue culture material. Purified LGA 391 and M150 virus when treated with 0.2% BPL for 18 hours at +4°C, also proved successful in that no detectable virus was recovered from in vitro or in vivo assays.

3.3.6.1. Kinetic rate of 0.2% BPL hydrolysis in various media

The kinetic rate of 0.2% BPL hydrolysis in relation to the rate of virus inactivation by BPL (Section 3.3.4) is demonstrated by Figs. 53 and 54 with LGA 391. At either temperature LGA 391 was inactivated before the half-life points of the drug. This also applies to all the Mopeia virus. Hydrolysis of BPL in human, guinea pig and monkey sera has shown that the half-life at 37°C was between 24-30 minutes and at 4°C 15-20 hours. Complete inactivation of LGA 391 and Mopeia virus was achieved within 30 minutes at 37°C and within 18 hours at +4°C. Total hydrolysis of BPL was completed within 3-4 hours at 37°C and within 3-4 days at +4°C.

TABLE 17. RATE OF HYDROLYSIS OF 0.2% BPL IN DIFFERENT MEDIA AT DIFFERENT TEMPERATURES

Temp°C	Media	Half-life	pH change	Complete hydrolysis
+4	PBS	36-40 hours	7.2 - 6.6	5 - 6 days
"	TCF	34-38 hours	7.4 - 6.2	5 - 6 days
"	Human sera	15-20 hours	8 - 6.5	3 - 4 days
"	Guinea pig sera	15-20 hours	7.8 - 6.4	3 - 4 days
"	Monkey sera	15-20 hours	7.8 - 6.3	3 - 4 days
37°C	PBS	60-65 minutes	7.2 - 6.5	5 hours
"	TCF	55-60 minutes	7.4 - 6.6	4½ hours
"	Human sera	24-30 minutes	8.0 - 6.3	2½ - 3 hours
"	Guinea Pig sera	24-30 minutes	7.8 - 6.0	2½ - 3 hours
"	Monkey sera	24-30 minutes	8.0 - 6.3	2½ - 3 hours

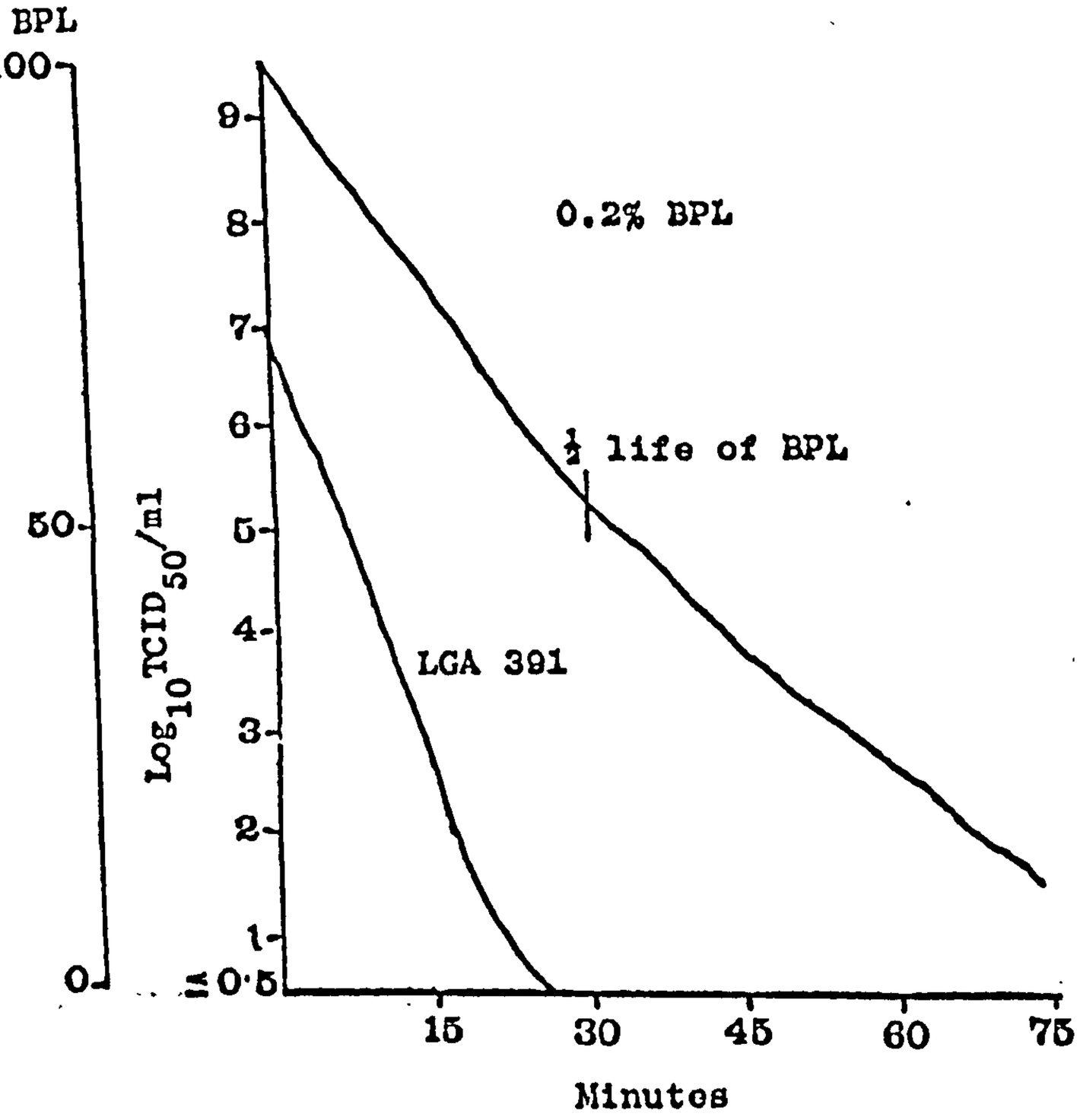


Fig. 53 Rate of LGA 391 inactivation and BPL hydrolysis in human sera at 37°C

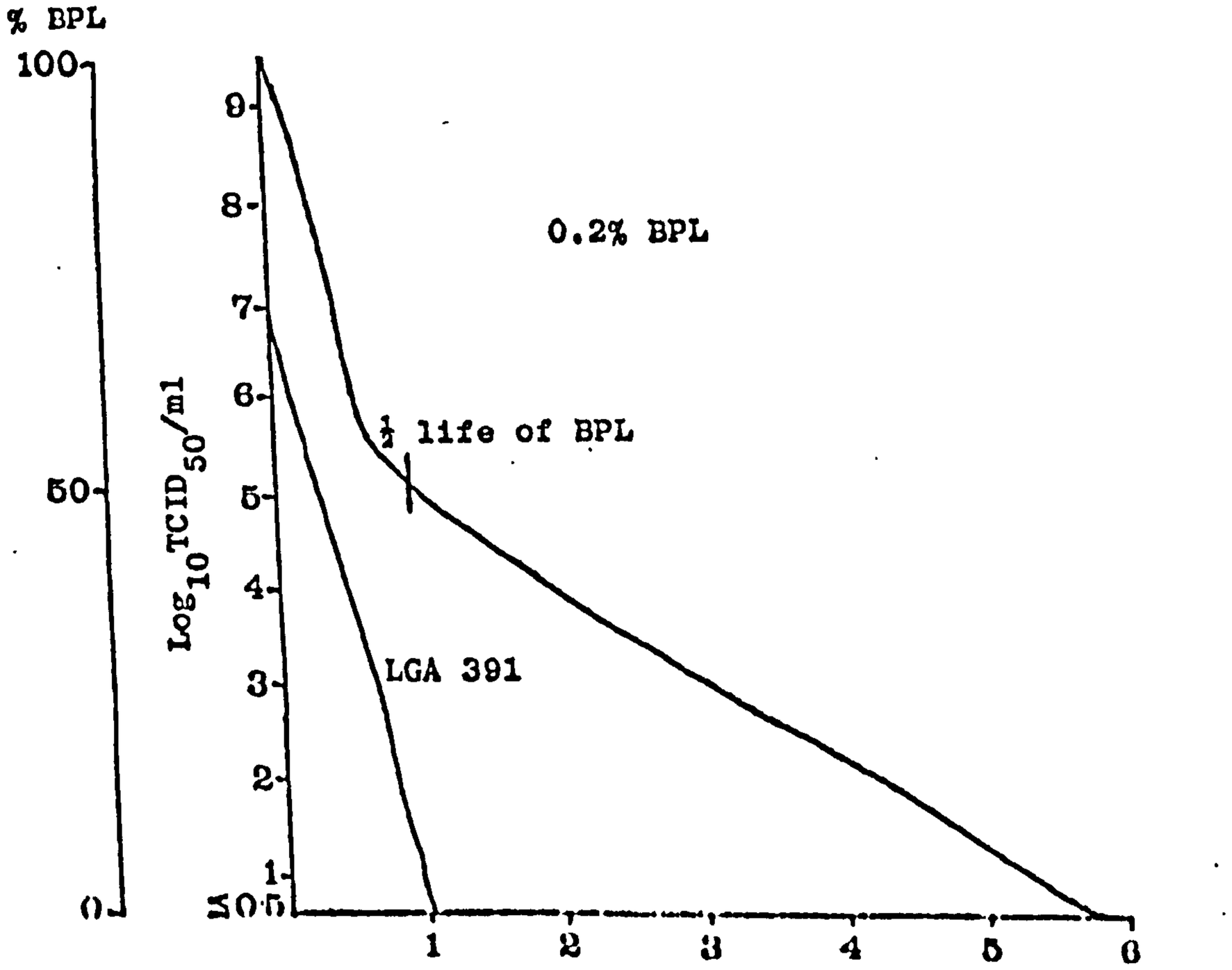


Fig. 54 Rate of LGA 391 inactivation and BPL hydrolysis in human sera at +4°C

In both PBS and tissue culture fluid containing 2% FCS the hydrolysis of BPL was very much slower at both 37°C and 4°C when compared to sera (Table 17). Inactivation of all viruses was completed well within the half-life of 0.2% BPL. Total hydrolysis was extended at +4°C by 1-2 days and at 37°C for 1-2 hours.

Measurements of the pH change during hydrolysis in both serum, PBS and TCF showed a marked reduction which also aided inactivation of the viruses.

3.3.6.2. BPL inactivation of infected Vero cells

The aim of this section was to determine the ability of BPL to inactivate intracellular viruses without affecting the cells ability to react to hyperimmune antisera in diagnostic immunofluoresence assays.

Infected Vero cells washed in PBS were resuspended in freshly prepared BPL solution (0.1% or 0.2%) in Plaisners medium pH 8.0 and kept at the times and temperatures shown in Tables 18 and 19. At the end of the reaction period cells were centrifuged, washed and suspended in PBS containing 0.01% bovine serum albumin (w/v) to an approximate final concentration of 10^6 per ml. About 0.02 ml was then placed on each spot of coated multispot microscope slides. Slides were air dried, fixed in cold acetone for 10 min, used at once or stored at -80°C in sealed plastic boxes. Samples of the inactivated cells were sonicated to disrupt the cells in a sonic bath and titrated in microassay plates. Indirect immunofluorescence readings performed on cells 10 days post-inoculation, were compared with those one hour post-inoculation. If the number of fluorescent foci after 10 days was the same as after one hour, the inoculated antigen was judged to be inactivated.

It was observed (Tables 18 and 19) that complete inactivation of the Lassa and Mopeia infected Vero cells with BPL was achieved, the antigenicity was retained and that hyperimmune sera reacted well with the

Table 18. BETAPROPIOLACTONE INACTIVATION OF VERO CELLS INFECTED WITH LGA 391 VIRUS

Virus	Exp. No.	Virus titre (- log)*	Cells/ml 10^6	BPL %	Contact time (min)	°C	Acetone Fixation 10 min	Inactivated	Reciprocal Ab. Titre	
									BPL Slides	Non-BPL inactivated Slides
LGA 391	1	5.1	± 1.0	0.2	2 x 10	22°	+	Yes	512	512
			± 1.0	0.2	2 x 10	22°	-	Yes	512	512
	2		± 1.0	0.1	2 x 10	22°	+	Yes	512	256
			± 1.0	0.1	2 x 10	22°	-	Yes	512	512
3			± 0.8	0.2	60 60	0° ** 37°	+	Yes	256	512
4			± 0.8	0.2	2 x 10	0°	+	Yes	256	256
			± 0.7	0.2	2 x 10	0°	-	Yes	256	256

*Represents intracellular virus concentration

**Cells exposed for 60 min at 0°C followed by 60 min at 37°C

Table 19. BETAPROPIOLACTONE INACTIVATION OF VERO CELLS INFECTED WITH MOPEIA VIRUS

Virus	Exp. No.	Virus titre (- log)*	Cells/ml 10^6	BPL %	Contact time (min)	°C	Acetone Fixation 10 min	Inactivated	Reciprocal Ab. Titre	
									BPL Slides	Non-BPL inactivated Slides
M 150	5	4.5	± 0.8	0.2	2 x 10	22°	+	Yes	256	256
			± 0.8	0.2	2 x 10	22°	-	Yes	256	256
	6		± 0.75	0.1	2 x 10	22°	+	Yes	256	256
			± 0.9	0.1	2 x 10	22°	-	Yes	256	128
	7		± 0.8	0.2	2 x 10	0°	+	Yes	128	256
			+ 0.8	0.2	2 x 10	0°	-	Yes	256	256
Z 478	8	5.6	± 0.9	0.2	2 x 10	22°	+	Yes	128	256
			± 0.85	0.2	2 x 10	22°	-	Yes	512	256
	9		± 0.9	0.1	2 x 10	22°	+	Yes	128	128
			± 0.85	0.1	2 x 10	22°	-	Yes	128	128
	10		± 0.8	0.2	2 x 10	0°	+	Yes	256	128
			± 0.75	0.2	2 x 10	0°	-	Yes	256	128

* Represents intracellular virus concentration

slide. No infectivity was demonstrated in any of the combinations used. It was noted, however, that the usable life of the unfixed slides was shortened on prolonged storage at -80°C.

3.3.7. Nucleic acid determination

Infectivity of all the Mopeia and LGA 391 viruses remained unchanged in the presence of BUDR, a deoxyribonucleic acid (DNA) inhibitor at any of the concentrations used (10^{-2}-10^{-5}). Effects of BUDR on the viruses are shown in Table 20. In the presence of BUDR, the development of a vaccinia (a DNA-containing virus) CPE was completely inhibited. A known RNA-containing virus, Echo-11, remained unaffected and developed a CPE.

TABLE 20. EFFECT OF 5-BROMODEOXYURIDINE ON GROWTH OF LGA 391 AND MOPEIA VIRUS IN VERO CELL CULTURE

Virus	Virus titre* BUDR (10^{-5}M)	No. BUDR
LGA391	6.8	7.0
M20410	6.6	6.5
M150	6.5	6.8
M152	7.1	7.2
Z478	5.6	5.8
Vaccinia	<1.0	6.2
ECHO-11	8.2	8.3

*Expressed as $Log_{10}TCID_{50}$/ml

3.3.8. Effect of Actinomycin D

The replication of Lassa and Mopeia viruses was inhibited by the transcription inhibitor, Actinomycin D at levels of greater than 1 µg/ml. The degree of inhibition at various dilutions of Actinomycin D is shown in Table 21.

TABLE 21. GROWTH OF LGA391 OR MOPEIA VIRUS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF ACTINOMYCIN D IN VERO CELL CULTURE

	Virus titre					
Actinomycin D	1.0	0.3	0.1	0.03	0.01	0
Virus						
LGA391	<0.5	1.5	4.5	5.0	6.8	6.8
M20410	"	0.5	3.4	4.7	5.0	5.2
M150	"	1.0	3.4	4.4	6.0	6.1
M152	"	1.0	2.8	5.2	6.0	6.5
Z478	"	0.5	2.9	4.2	6.0	6.4

Vero cells infected with Lassa and Mopeia viruses in the presence of Actinomycin D and removed after absorbtion showed normal growth of virus. Thus, the suppression of Lassa and Mopeia virus replication by Actinomycin D was found not to be due to the direct effect of the drug upon the viruses.

3.4 ANTIGENIC CROSS-REACTIONS

3.4.1. Serological reactions using polyclonal antisera

The basic pattern of cross-reactions amongst Mopeia and LGA 391 viruses by complement-fixation test demonstrated a fair degree of uniformity. Low titre CFT cross-reactions with LCM was a consistent finding. The extent of cross-reaction between Mopeia and Lassa strains was demonstrated with guinea pig sera as shown in Table 22.

TABLE 22. CROSS-REACTIONS AMONG LGA 391 AND MOPEIA VIRUS BY COMPLEMENT FIXATION TEST

	Guinea Pig Serum[1]					
Antigen	LGA391	M20410	M150	M152	Z478	LCM
LGA391	64	32	32	64	32	8
M20410	64	32	16	32	16	4
M150	64	16	128	32	32	4
M152	64	32	16	64	32	4
Z478	64	32	16	ND	64	8
LCM[2]	16	8	8	8	8	128

[1]Reciprocal of serum CF titre

[2]LCM antigen was mouse brain antigen

ND - Not done

Extensive studies using CFT's were avoided in this study because of the time-consuming and dangerous aspect of antigenic preparation. During the preliminary studies anti-complementary activity was often encountered in serum specimens obtained from mice, guinea pigs and monkeys. Furthermore, IF antibodies were detected earlier in all the experimental studies and proved a more reliable test in diagnosing and monitoring the disease process.

Using the IFA test all Lassa and Mopeia viruses demonstrated a similar staining pattern for virus-specific antisera. The main features were in the infected cell cytoplasm, where bright yellow-green inclusion bodies varied in shape and size, from sand-like granules to large odd-shaped clumps of antigen. The brightness of the staining and percentage of stained cells usually diminished as the serum was diluted. The serum dilution which showed slightly diminished brightness of staining in the majority of infected cells was chosen as an endpoint.

Guinea pig serum, mouse ascitic fluid or serum, and Rhesus monkey serum prepared against the Mopeia strains all cross-reacted with each other and with LGA 39.1 demonstrating little variation. They did not react with LCM or Pichinde antigens (Table 23). Cross-reactions using convalescent sera from six human cases of Lassa (Table 24) reacted with all Mopeia viruses, two reacting with LCM at low titre but not with Pichinde. Overall, animal immune sera cross-reacted more extensively than human convalescent serum. Normal human and animal sera were included in the tests with no staining observed with any of the antigens.

TABLE 23.

RECIPROCAL FLUORESCENT ANTIBODY TITRES OF ANIMAL CONVALESCENT SERA AND ASCITIC FLUIDS FOR ARENAVIRUS ANTIGENS

Antigen	LGA391	M20410	M150	M152	Z478
			Guinea pig serum		
LGA391	2048	512	128	1024	128
M20410	128	1024	512	512	512
M150	1024	512	2048	256	512
M152	1024	1024	4196	4196	512
Z478	512	64	128	128	512
LCM	<4	<4	<4	<4	<4
Pichinde	<4	<4	<4	<4	<4
			Mouse ascitic fluid		
LGA391	256	32	128	ND	64
M20410	32	128	256	ND	128
M150	64	64	512	ND	256
M152	128	128	256	ND	256
Z478	32	128	256	ND	512
LCM	<4	<4	<4	ND	<4
Pichinde	<4	<4	<4	ND	<4
			Rhesus Monkey Serum		
LGA391	2048	1024	2048	4196	2048
M20410	512	4196	1024	2048	2048
M150	1024	2048	4196	2048	2048
M152	1024	512	2048	4196	2048
Z478	512	1024	2048	2048	2048
LCM	<4	<4	<4	<4	<4
Pichinde	<4	<4	<4	<4	<4

TABLE 24. RECIPROCAL FLUORESCENT ANTIBODY TITRES OF HUMAN CONVALESCENT SERA AGAINST ARENAVIRUS ANTIGENS

	Serum antibody titre after Lassa virus infection					
Antigen	G.A.	J.W.	P.B.	T.E.	G.L.	A.W.
LGA391	1024	256	4096	128	128	512
M20410	256	64	2048	32	16	128
M150	512	128	2048	16	32	64
M152	512	128	2048	64	64	128
Z478	256	64	1024	32	32	512
LCM	16	<8	16	8	8	<8
Pichinde	<8	<8	<8	<8	<8	<8

On the basis of the neutralization test used it would appear that Lassa convalescent antisera from both experimental animal and human infections gave rise to neutralisation antibodies to LGA 391. A range of human antiserum used in this exercise did not react with any of the Mopeia viruses (Table 25). The reproducibility of Lassa convalescent antiserum reacting with Mopeia viruses could not be determined with the Rhesus monkey as all monkeys infected with LGA 391 did not survive long enough to develop neutralization antibodies. Convalescent sera obtained from Mopeia infected Rhesus monkeys did not react with LGA 391, although they demonstrated varying degrees of cross-reactivity amongst themselves (Table 26). Guinea pig anti-Lassa convalescent sera (120 days) demonstrated low neutralizing cross-reactivity against all the Mopeia viruses studied (Table 27). However, guinea pig antisera prepared against the Mopeia viruses did not neutralize LGA 391, but showed limited cross-reactivity amongst themselves.

TABLE 25. CROSS-REACTIVE NEUTRALIZING ANTIBODIES FOUND IN HUMAN CONVALESCENT SERA AGAINST ARENAVIRUS ANTIGENS

	Neutralization Index					
Sera*	G.A.	J.W.	P.B.	T.E.	G.L.	A.W.
Antigen						
LGA391	2.1	1.0	2.3	0.9	0	0
M20410	0	0	0	0	0	0
M150	0	0	0	0	0	0
M152	0	0	0	0	0	0
Z478	0	0	0	0	0	0
LCM	0	0	0	0	0	0
Pichinde	0	0	0	0	0	0

* All serum used was 3 months post Lassa infection

TABLE 26. CROSS-REACTIVE NEUTRALIZING ANTIBODIES FOUND IN MONKEY ANTI-MOPEIA CONVALESCENT SERA (60 DAYS)

	Neutralization Index			
Sera	Z478	M150	M152	M20410
Antigen				
LGA391	0	0	ND	0
M20410	0.8	1.8	2.0	1.6
M150	0.7	2.0	2.1	1.8
M152	1.8	0.9	2.3	1.0
Z478	1.8	ND	1.6	0.6

TABLE 27. CROSS-REACTIVE NEUTRALIZING ANTIBODIES FOUND IN GUINEA PIG ANTI-MOPEIA/LASSA CONVALESCENT SERA (120 DAYS)

	Neutralization Index				
Sera / Antigen	Z478	M150	M152	M20410	LGA 391
LGA391	0	0	0	0	2.8
Z478	1.7	1.5	2.0	1.0	0.9
M150	1.2	2.5	2.0	2.0	0.8
M152	0.8	1.8	2.0	1.8	1.3
M20410	0.8	1.3	2.0	2.2	0.5

3.4.2. Serological reactions using monoclonal antibodies to LGA 391

Seventy-eight hybridoma cell lines producing antibody to LGA 391 were established. Screening by immunofluorescence revealed antibodies that reacted in a granular pattern with antigens expressed only in the cytoplasm of infected cells and those reacting with determinants expressed in the cytoplasm and at the surface of infected cells. Frequency of occurrence of virus-specific antibody-producing hybridomas varied from 10 - 50% of wells showing growth in eight independent fusions. Due to the number of hybridomas produced and time involved, details have had to be confined to only a few of the hybridomas.

Analysis of 20 monoclonal antibodies for their immunoglobulin class - specificity was determined by immunodiffusion against monospecific reagents. This revealed six IgG1, 19 IgG2A and one unknown reactor.

LGA 391 contains three major polypeptides, a prominent protein of molecular 60,000, which corresponds to the nucleocapsid protein N and two glycoproteins of molecular weight 45,000 and 38,000 G1 and G2 respectively (Clegg and Lloyd 1982). It was of interest to determine which polypeptides were recognised by the monoclonal antibodies under study. This determination was considered useful if the antibodies were to be used

as molecular probes to study replication and antigen expression. Immune precipitation analysis using radioactive labelled LGA 391 was performed on 20 of the monoclonal antibodies. All were found to immunoprecipitate viral nuclear protein (Table 28).

Cross-reactivity of LGA 391 virus-specific hybridoma antibodies with heterologous arenaviruses of the 'Old World' (Lassa and Mopeia) and 'New World' (Tacaribe complex) viruses was assessed by IF on acetone fixed infected cells. Seven LGA 391 monoclonal antibodies were studied in detail and reacted with Junin (XJ3), Pichinde (COAN R 6053), Tacaribe (TVL-11573), Lymphocytic choriomeningitis (ARM: CA 1371), LGA 391, M150, M152, M20410 and Z478 viruses (Table 28). Of these seven monoclonals four (No. 52, 63, 72 and 79) were specific for LGA 391 and did not react with any of the Mopeia viruses (Table 28). This specificity to Lassa viruses as a whole has been extended to include several human and Mastomys isolates originating from Liberia, Nigeria and Sierra Leone by both immunofluorescence and 'Western blot' analysis (Section 2.6.3.). None of the monoclonals reacted with Junin, Pichinde, Tacaribe or LCM.

3.4.3. Cross-reactivity between arenaviruses using monoclonal antibodies to other arenaviruses

Cross-reactivity of Mopeia, LCM, Pichinde, Tacaribe and Junin virus-specific hybridoma antibodies with heterologous arenaviruses of the 'Old World' and 'New World' groups was assessed by indirect immunofluorescence. Four M20410 virus-specific hybridoma antibodies tested were either IgG2A and nucleo protein specific. Each reacted with the 'Old World' viruses and not the 'New World' viruses of Junin or Pichinde. Neither did they react with LCM. Monoclonal 53-29-1 was specific for the Mopeia viruses and did not react with LGA 391. Monoclonal 52-153-2 was specific for LGA 391 and reacted very poorly with the Mopeia viruses (Table 29).

TABLE 28. HYBRIDOMA ANTIBODIES TO LGA 391 VIRUS

Reference No.	Immuno-globulin Subclass	Lassa polypeptide Specificity	IFA reactions with other arenaviruses							
			Junin	Pichinde	LCM	Z478	LGA391	M150	M152	M20410
35 (TC)	IgG1	N	<4	<4	<4	32	1024	256	512	512
41 (TC)	IgG2A	N	<4	<4	<4	16	512	16	32	32
52 (TC)	IgG2A	N	<4	<4	<4	<8	512	8	<8	<8
63 (TC)	IgG	N	<4	<4	<4	<8	128	<8	<8	<8
65 (TC)	IgG1	N	<4	<4	<4	32	64	32	64	128
72 (TC)	IgG2A	N	<4	<4	<4	<4	128	<8	<8	<8
79 (TC)	IgG2A	N	<4	<4	<4	<8	2048	<8	<8	<8

TABLE 29. HYBRIDOMA ANTIBODIES TO MOPEIA VIRUS (M20410)

Reference No.	Immuno-globulin Subclass	Mopeia polypeptide Specificity	IFA reactions with other arenaviruses							
			Junin	Pichinde	LCM	Z478	LGA391	M150	M152	M20410
53-29-1 (MAF)	IgG2A	N	<10	<10	<10	12,800	<10	25,660	12,800	102,400
52-153-3(MAF)	IgG2A	N	<10	<10	<10	<10	640	<10	40	<10
52-54-6 (MAF)	IgG2A	N	<10	<10	<10	3,200	1,600	6,400	12,800	6,400
53-237-5(MAF)	IgG2A	N	<10	<10	<10	<10	<10	<10	40	40

TABLE 30. HYBRIDOMA ANTIBODIES TO TACARIBE VIRUS (TVL-11573)

Reference No.	Immuno-globulin Subclass	Tacaribe Polypeptide Specificity	IFA reactions with other arenaviruses				
			Junin	Pichinde	LCM	LGA391	Mopeia strains
2-100-3	IgG1	N	+	-	-	-	-
2-16-2	IgG2A	N	+	+	-	-	-
2-24-9	IgG2B	N	-	-	-	-	-
2-30-10	IgG2A	N	-	-	-	-	-
2-31-4	IgG2A	N	-	-	-	-	-
2-69-9	IgG2A	N	-	-	-	-	-
2-52-2	IgG2A	N	+	-	-	-	-
2-74-9	IgG2A	N	+	-	-	-	-
2-84-11	IgGM	N	-	-	-	-	-
2-48-3	IgG2A	N	+	+	-	-	-

TABLE 31. HYBRIDOMA ANTIBODIES TO JUNIN VIRUS (XJ3)

Reference No.	Immuno-globulin Subclass	Junin polypeptide Specificity	IFA reactions with other arenaviruses				
			Tacaribe	Pichinde	LCM	LGA391	Mopeia strains
3-88-7	IgG2A	N	+	-		-	-
3-46-8	"	N	+	-		-	-
3-46-1	"	N	+	-		-	-
3-6-2	"	N	+	-		-	-
3-70-11	"	N	+	-		-	-
3-67-7	"	N	+	-		-	-

TABLE 32. HYBRIDOMA ANTIBODIES TO LYMPHOCYTIC CHORIOMENINGITIS (LCM)
(ARM, CA 1371, Rowe et al., 1963)

Reference No.	Immuno globulin Subclass	LCM polypeptide Specificity	IFA reactions with other arenaviruses							
			Junin	Pichinde	LCM	LGA391	M150	M152	M20410	Z478
1-1-3 (MAF)	IgG 2A	NP	<4	<4	12,800	400	400	200	400	200
24-A-21 (MAF)	"	"	<4	<4	51,200	51,200	12,800	25,600	51,200	6,400
25-3-9 (MAF)	"	"	<4	<4	12,800	6,400	3,200	12,800	6,400	3,200
*9-7-5 (MAF)	"	GP-2	<4	<4	800	<2	16	200	400	200

*Surface IF reaction on unfixed antigen infected cells

TABLE 33. HYBRIDOMA ANTIBODIES TO PICHINDE VIRUS (COAN R 6053)

Reference No.	Immuno globulin Subclass	Pichinde polypeptide Specificity	IFA reactions with other arenaviruses							
			Junin	Pichinde	LCM	LGA391	M150	M152	M20410	Z478
75-7 (MAF)	IgG2B	N	<4	10,000	512	8	512	512	512	<4
75-19 (MAF)	IgG2A	N	<4	8,000	<4	<4	<4	<4	2,048	256
75-23 (MAF)	IgGG3	N	<4	4,000	<4	1,024	10	1,024	10	<4
75-25 (MAF)	IgG1	ND	<4	20,000	<4	<4	<4	<4	<4	128

The monoclonal antibodies produced from Junin and Tacaribe hybridomas did not react with any of the Lassa or Mopeia strains. Of the 10 Tacaribe monoclonals only five reacted with Junin and of these two (2-16-2 and 2-43-3) reacted with Pichinde. Five nuclear protein reactive monoclonals were specific for only Tacaribe virus (Table 30). All six Junin monoclonals reacted with Tacaribe but not Pichinde (Table 31).

The four monoclonal antibodies derived from LCM hybridomas (kindly supplied by Dr Buchmeier, Scripps Clinic, California) consisted of three nucleoprotein specific antibodies and one glycoprotein-2 directed antibody (9-7-5). None of the monoclonals reacted with any of the 'New World' viruses tested (Table 32). This confirmed the published work of Buchmeier et al., (1981). However, the use of 9-7-5 did not completely differentiate between the Lassa and Mopeia viruses as was indicated in the same publication.

Forty-two monoclonal antibodies to Pichinde virus were tested against LGA 391 and Mopeia infected cells. Four of these antibodies reacted with one or more of the Lassa or Mopeia viruses (Table 33). These demonstrate a reaction with both 'Old' and 'New World' viruses.

3.5. COMPARISON OF IMMUNOCHEMICAL PROPERTIES OF LASSA AND MOPEIA VIRUS STRUCTURAL PROTEINS

LGA 391 virus had three major proteins of molecular weight 60,000 (N), 45,000 (G1) and 38,000 (G2) (Clegg and LLoyd, 1983) and was similar if not identical to those corresponding to the Mopeia virus proteins except that all the Mopeia virus strains showed a large N protein of molecular weight of 62,000.

Proteins of LGA 391 and Mopeia viruses separated on SDS-polyacrylamide gels, transferred to nitrocellulose were detected by binding antibodies of guinea pig and Rhesus monkey sera raised against LGA 391 or Mopeia viruses. When incubated with antisera to LGA 391, antibody bound to the N and G2 proteins of Lassa and Mopeia strains tested (Fig. 55a).

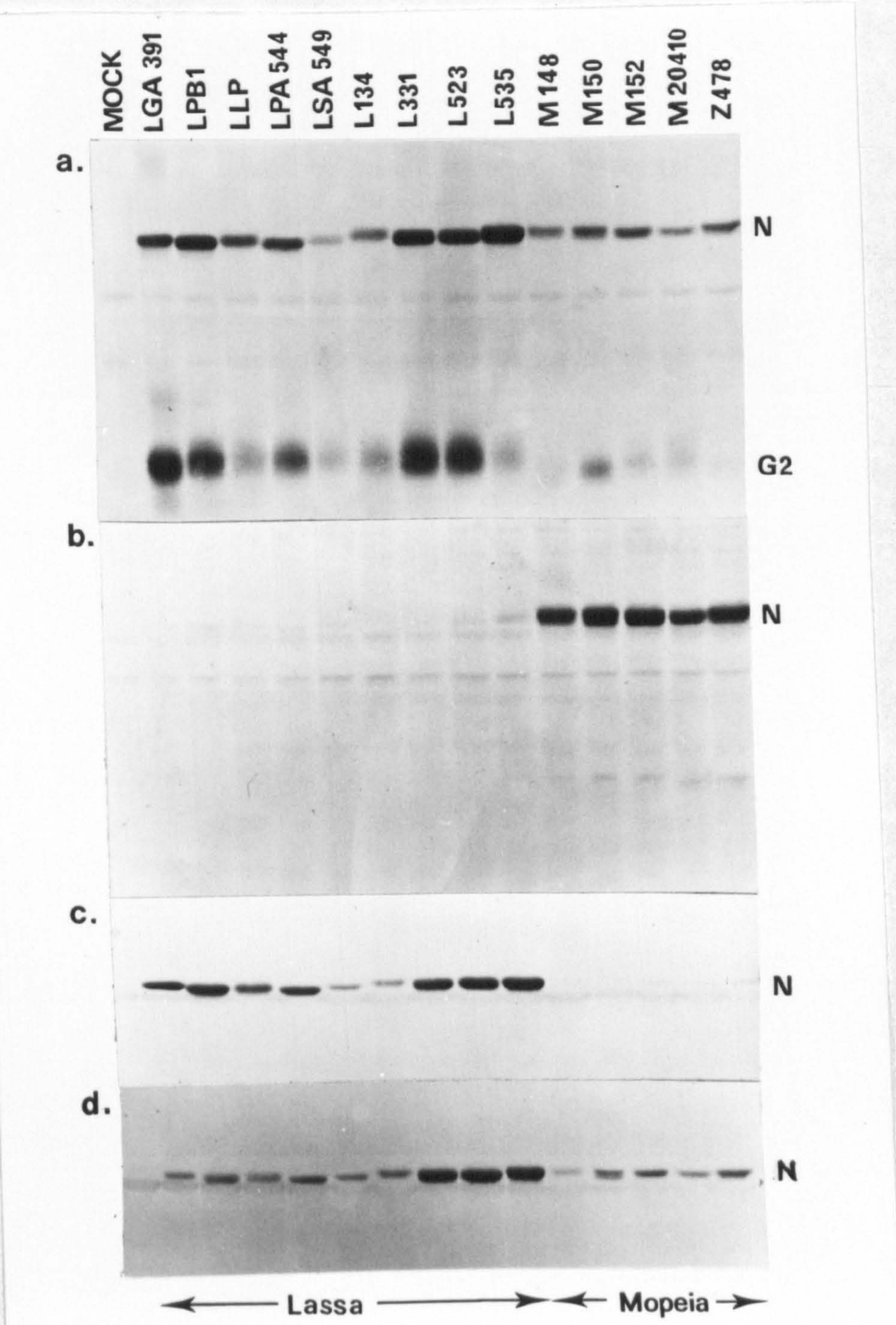


Fig. 55. Proteins of various strains of Lassa and Mopeia viruses. Cell lysates were made 2 days after infection of CV-1 cells with strains of Lassa or Mopeia viruses, and the proteins separated on 10% polyacrylamide gels and transferred to nitrocellulose sheets. The transfers were incubated with (a) guinea pig anti-Lassa virus antisera (LGA 391), (b) guinea pig anti-Mopeia virus (M148), (c) monoclonal antibody 52 (raised against LGA 391) and (d) monoclonal antibody 35 (raised against LGA 391), followed by the appropriate peroxidase-conjugated second antibody.

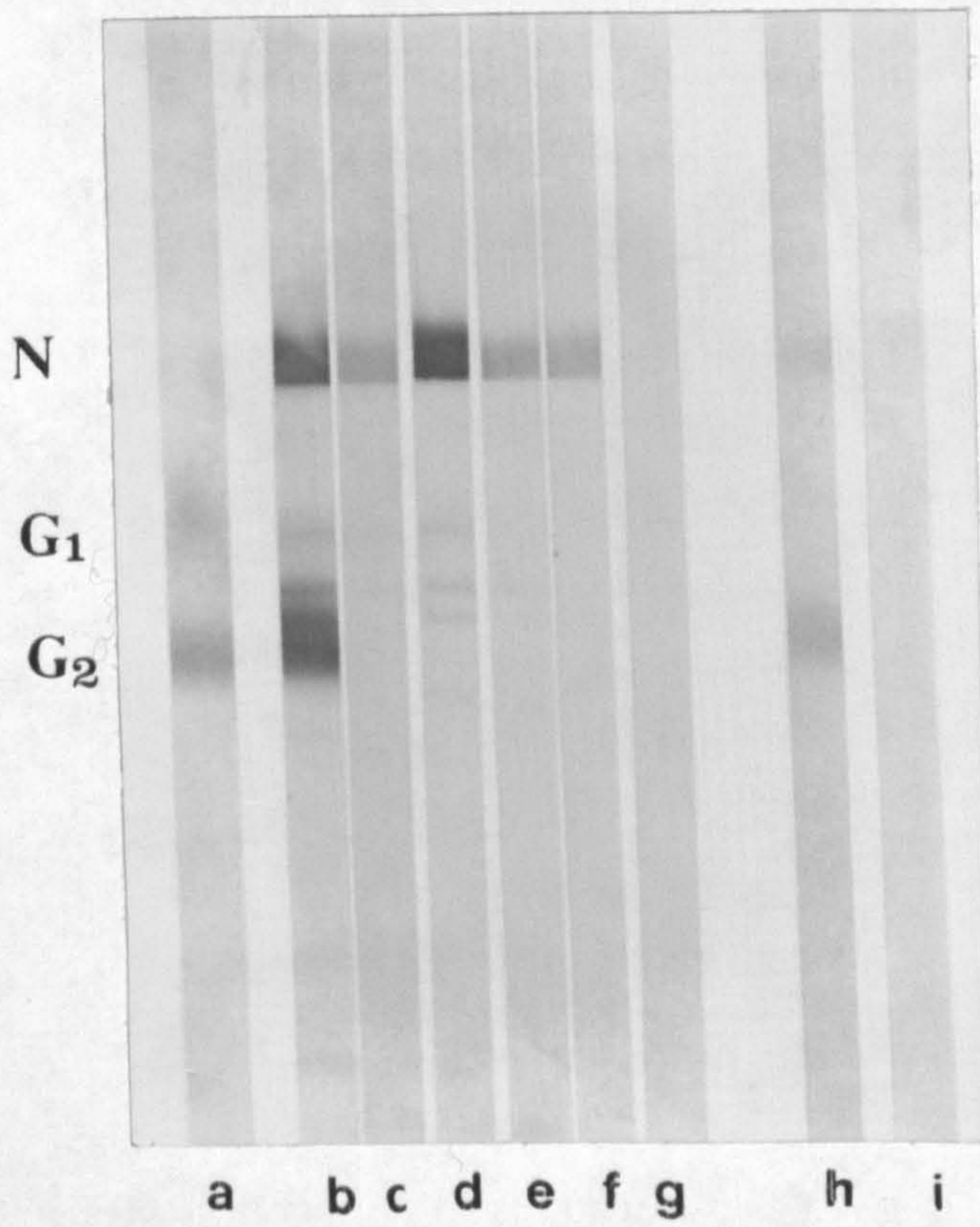


Fig.56 Cross-reaction between SDS gel electrophoresis proteins of purified Lassa virus with G.P. anti-Mopeia serum. Purified virus was disrupted in 1% SDS, 1% mercaptoethanol and analysed on 9-15% polyacrylamide gradient gels 1.5 mm thick. Tracks were strips cut from 3.5 cm slots containing 15 µg protein after transfer to nitrocellulose. (a) incubated with concanavalin A, (b-i) incubated 4 hours with guinea pig antiserum to (b) LGA 391, (c) M148, (d) M150, (e) M152, (f) Z478, (g) M20410, (h) human anti-Lassa (strain GA) (i) normal guinea pig.

Alternatively when incubated with antisera to any of the Mopeia viruses, antibody bound strongly to the N protein only of Mopeia strains but did not react or at most reacted weakly to all the West African Lassa strains (Fig. 55b). (N.B. All the L designated strains on Fig. 55 are different Lassa isolates from West Africa.) No G2 reaction was noted in Fig. 55b. No conclusion can be drawn concerning G1 glycoprotein because of its lack of antigenicity in the "Western blot" system.

The LGA 391 nucleoprotein directed monoclonals were also found to bind to the nucleoproteins of Lassa and Mopeia viruses in characteristic and differentiating patterns (Fig. 55 c and d). This data exactly supports the pattern of differentiation observed by immunofluorescent staining of virus-infected cells (Table 28).

Finally it should be noted that "Western blots" using purified LGA 391 confirms that anti-Mopeia serum does react with Lassa N protein, demonstrating the existence of common antigenic determinants (Fig. 56).

3.6. TRANSMISSION OF LASSA AND MOPEIA VIRUSES TO MICE

Tissue culture pools grown in Vero cells and passed three times (VCP3) were inoculated into male and female Porton strain mice at different ages and by different routes. The volume of each inoculum for i.p. was 0.1 ml and for i.c. 0.03 mls.

3.6.1. Susceptibility

The susceptibility of mice at different ages and routes of infection are shown in Table 34. No difference was observed between male and female mice.

TABLE 34. EFFECT OF LGA 391 AND MOPEIA VIRUSES ON PORTON STRAIN MICE AT DIFFERENT AGES AND BY DIFFERENT ROUTES OF INFECTION

	148	150	152	LGA 391	478
I.C.					
2 - 4 days	±	±	-	-	±
10 days	-	-	±	±	-
14 days	+	+	+	+	+
21 days	+	+	+	+	+
I.P.					
2 - 4 days	-	-	- +	- +	-
10 days	-	-	-	-	-
14 days	+	-	-	-	-
21 days	-	-	-	-	-

\+ No survivors

± Sick, paralysed or stunted growth sometimes leading to death

\- No illness

Viruses M152 and LGA391 proved fatal to 2-4 week old mice within 9-12 days post infection with doses ranging from 10^2-10^8 $TCID_{50}$/0.03 mls when introduced i.c. Mopeia viruses M 148, M150 and Z478 also proved fatal to 2-4 week old mice between 7-9 days post infection. Suckling mice were not fatally affected when inoculated by the i.c. route (Table 35).

TABLE 35. SUSCEPTIBILITY OF PORTON STRAIN MICE (2-4 WEEKS) TO LGA 391 AND MOPEIA VIRUSES WHEN INTRODUCED BY THE I.C. ROUTE OF INFECTION

Virus	Dose $Log_{10}TCID_{50}$	Day	% died
LGA391	8	D13+	20
	5	D13+	60
	4	D11	100
	2	D11	100
	1	D12+	50
M20410	7	D9	100
	5	D8	100
	3	D12	100
	1	D14+	40
M150	8	D7	100
	6	D7	100
	4	D12+	0
	2	D14+	40
M152	8	D9	100
	6	D9	100
	4	D7	100
	2	D11+	50
Z478	8	D9	100
	6	D7	100
	4	D10+	80
	2	D14+	60

Infection by the i.p. route had no fatal effect on adult or suckling mice at any age. Blind passage of brain homogenates derived from infected suckling mice did not alter the data.

3.6.2. Clinical observations

During the course of a fatal infection following i.c. inoculation, a reduced intake of food and water was the first sign of illness. The body weight gradually decreased throughout the course of the infection. During this phase of the infection the mice became immobile, had an increased respiratory rate, and their rear limbs demonstrated extensive tremors which finally resulted in paralysis.

During the course of a non-fatal illness the suckling and adult mice developed milder clinical signs. The main feature appeared to be a reduction in body hair and weight during the first 14 days post infection, followed by increase in body weight at half the normal rate.

3.6.3. Virus levels in blood and tissue

The comparative virus levels in the blood of mice infected with 3 $\log_{10}TCID_{50}$ of virus by i.p. and i.c. routes with Lassa and Mopeia strains are shown in Figs.57 and 58.

In all cases virus was first detected on day 2 p.i. by the i.c. route and on day 4 by the i.p. route. With all the viruses studied, maximum levels of virus developed by day 4 p.i. i.c. and days 6-8 p.i. i.p. . After i.c. infection, virus levels of between 10^4-$10^5/TCID_{50}$/ml were observed with M152, Z478 and LGA 391 and 10^2-$10^3 TCID_{50}$/ml with M150 and M20410 all of which persisted until death.

Through the non-fatal i.p. route, M150, Z478 and M20410 virus levels in the blood reached 2-3 $\log_{10}TCID_{50}$/ml while those of M152 and LGA 391 reached 4 $\log_{10}TCID_{50}$/ml. In all cases virus levels were maintained for 4 days. By ten days p.i. no virus was detectable in the blood. In the case of LGA391 and M152 virus was detected in the urine at day 30 although this was not a consistent finding.

In all fatal infections virus was detected in all major organs of the body at levels greater than in the blood (Fig.59).

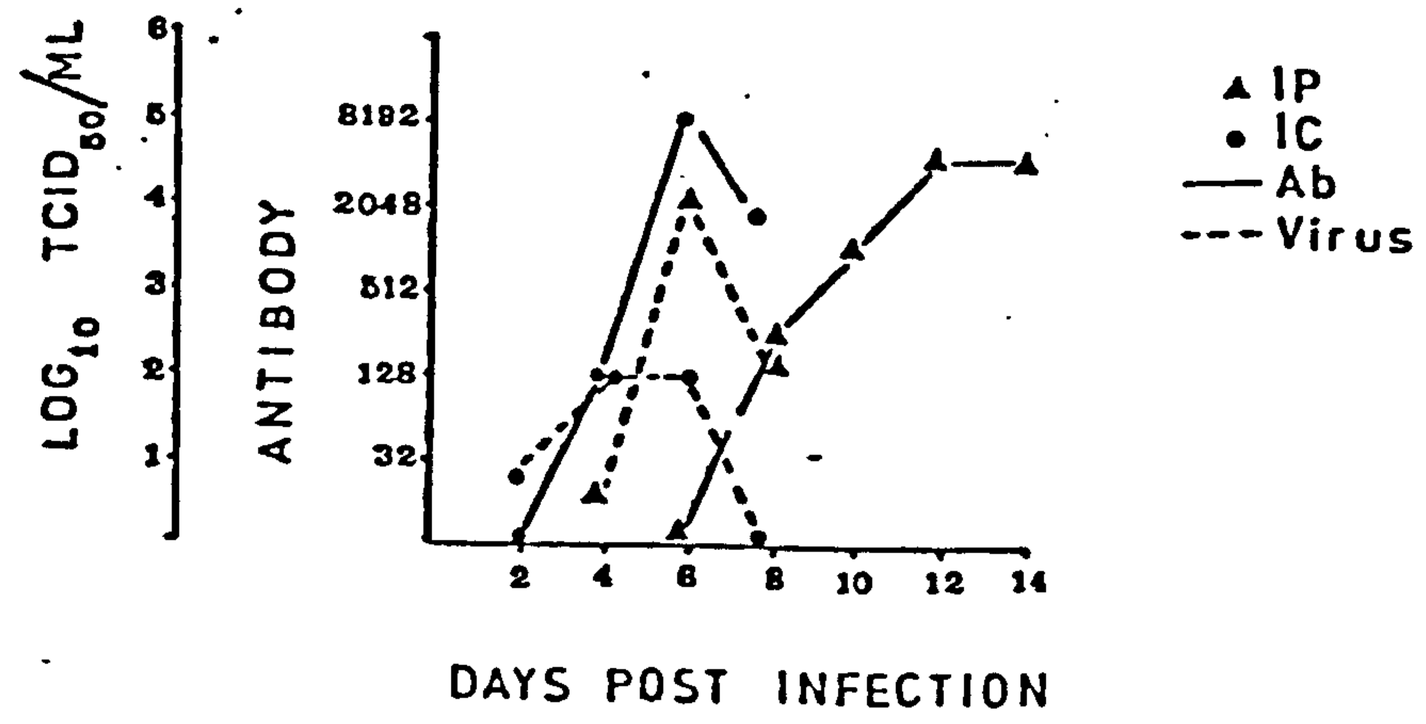


Fig.57. Viraemia and immunofluorescent antibody (IgG) responses in Porton mice inoculated with 3 $\log_{10}$ $TCID_{50}$ Lassa or Mopeia viruses by either the I.C. or I.P. route.

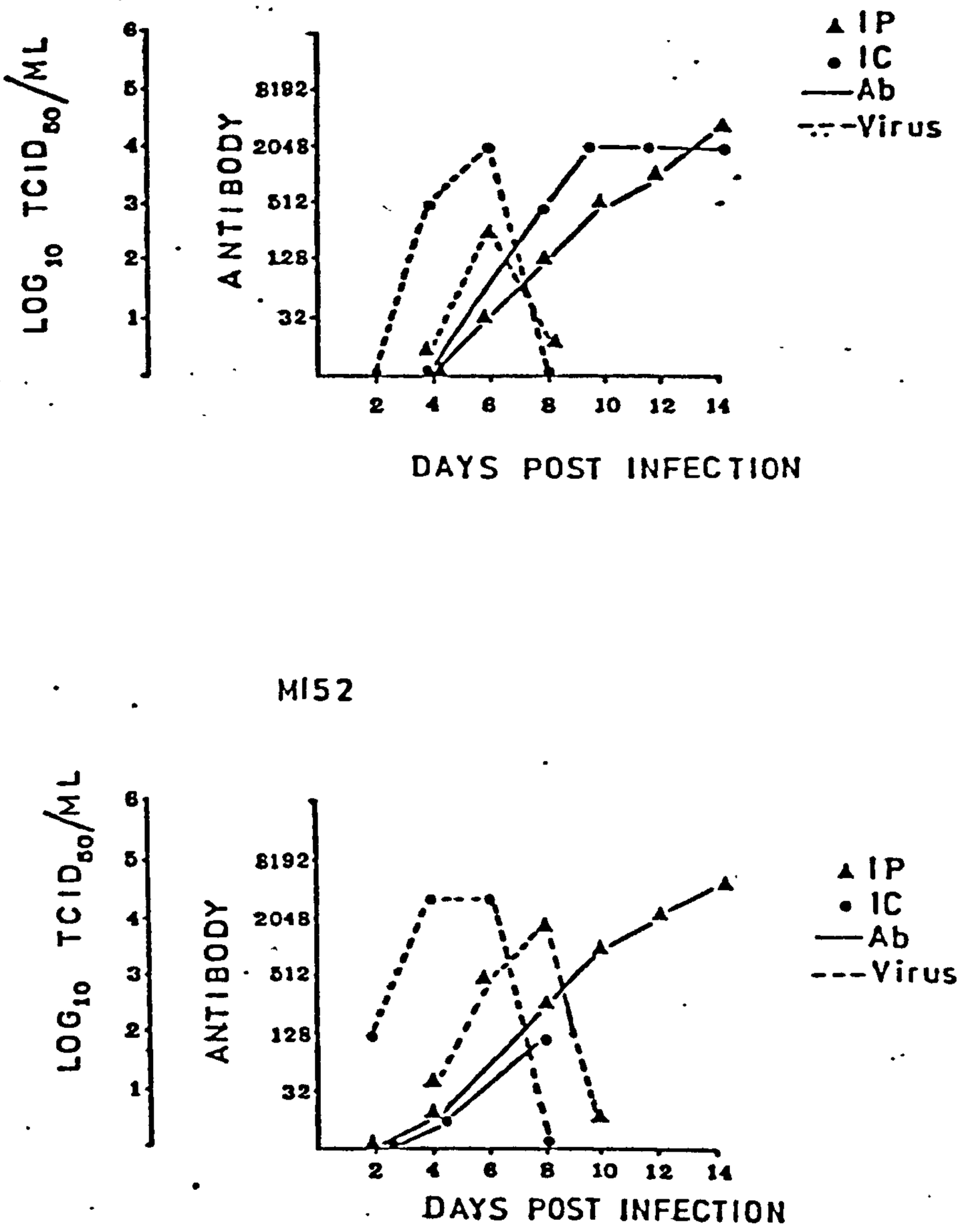
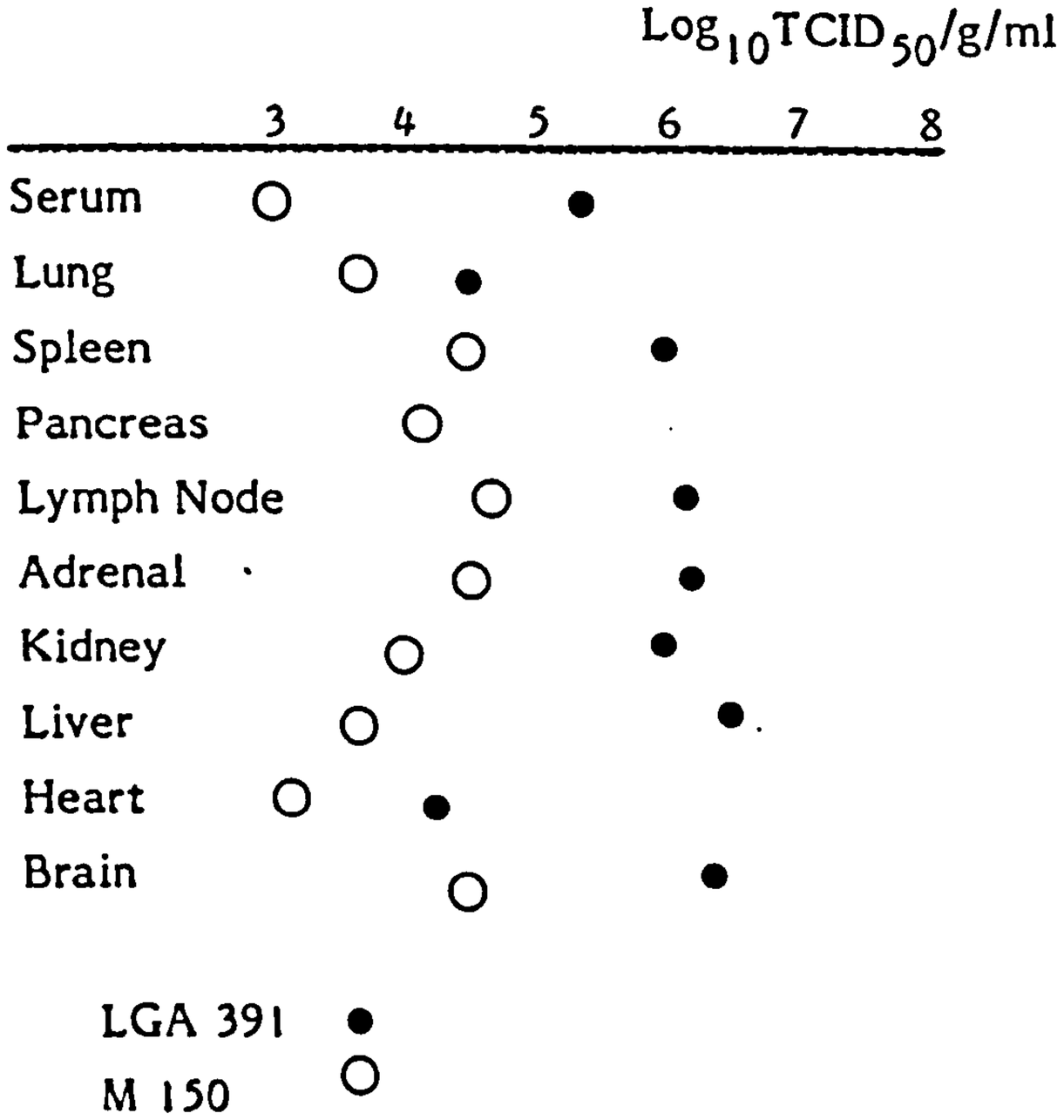


Fig.58: Viraemia and immunofluorescent antibody responses in Porton mice inoculated with 3 $\log_{10}$ $TCID_{50}$ Mopeia viruses by either the I.C. or I.P. route.

Fig. 59 INFECTIOUS LGA 391 AND MOPEIA VIRUS CONCENTRATIONS RECOVERED FROM SERA AND TISSUES OF MORIBUND 2-4 WEEK ADULT PORTON STRAIN MICE OBTAINED 7-10 DAYS AFTER I.C. INOCULATION WITH 3 LOG_{10} $TCID_{50}$



A similar pattern to that shown by Fig. 59 was found with M20410, M152, and Z478 viruses.

3.6.4. Histopathology

Mice inoculated by the i.p. route with LGA 391, M152, M150, M20410 and Z478 demonstrated no histological lesions in any organ despite the extensive virus recovery. The response to the i.c. route of infection demonstrated widespread brain lesions in mice between days 7 and 14 with all viral infections, LGA 391 and M148 viruses causing the most severe lesions. M150 and Z478 were as virulent although the brain was not so severely affected. Brain lesions included a variable degree of lymphocytic meningitis and perivascular cuffingand in some cases the choroid plexus was infiltrated by lymphocytes. Other organs were not affected.

3.6.5. Clinical Biochemistry

To evaluate the significance of altered serum enzymes and substrate values during infection, normal values were first established from the Porton mouse model. The normal values are tabulated in Table 36.

TABLE 36. NORMAL VALUES FOR SOME SERUM ENZYMES AND SUBSTRATES IN PORTON MICE*

Enzyme/Substrate	Normal value	±SE	Units
GOT	79.8	14.7	IU/l
GPT	59.8	4.42	"
LDH	2600	68.2	"
α-HBDH	1170.1	64.4	"
CK	100	9.5	"
γ-GT	ND	-	-
AP	125	14.4	mg/100 ml
Creatine	0.12	0.03	"
Urea	186.8	21.14	
Total protein	ND	-	-
Triglycerides	ND	-	-
Cholesterol	ND	-	-

*Each value represents the arithmetic mean of 30 samples.

Both serum glutamic oxaloacetic transaminase (GOT) and pyruvic transaminase (GPT) are elevated during Lassa and Mopeia infections of adult mice initiated by the i.c. and i.p. routes. Peak GOT levels are reached on day 6 p.i. of a fatal i.c. infection falling to normal by day 8 p.i. (Fig. 60) after which death occured. In the case of the non-fatal i.p. infection a moderate rise in activity was demonstrated over the first 6 days p.i. gradually returning to normal by day 14 p.i. GPT activity after both Lassa and Mopeia infections showed marked increases, reaching a peak by day 5 p.i. and dramatically decreasing to normal on day 6 p.i.

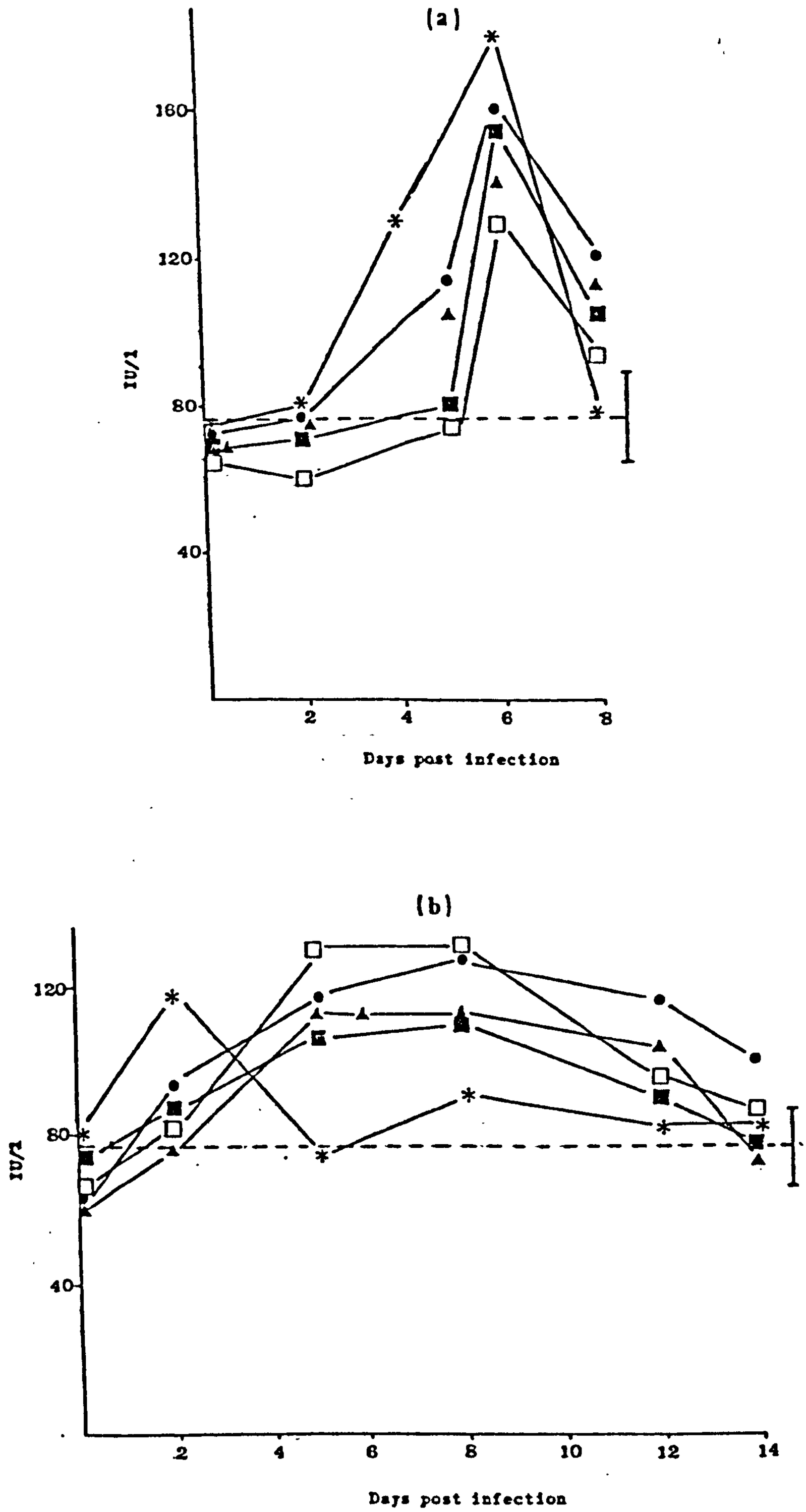
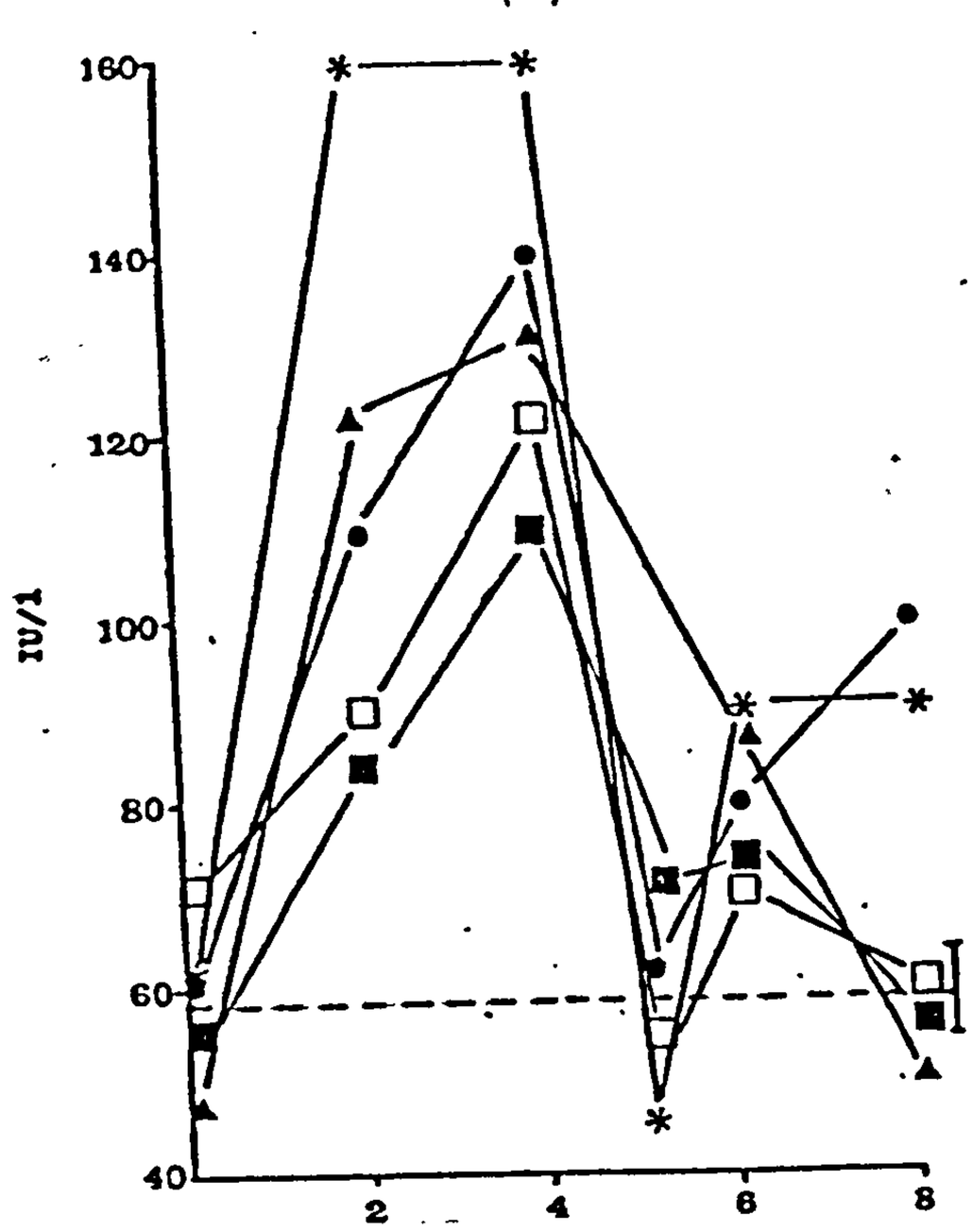


Fig. 60 Glutamine oxaloacetate transaminase (GOT) activity in the sera of Porton strain mice (3 wks) inoculated (a) i.c., (b) i.p. with 3 $\log_{10}$ $TCID_{50}$ of Lassa and Mopeia viruses. Points are arithmetic means of 20 mice

●: LGA391 ▲M150 □M152 ■20410 *Z478

--------I Normal range



(b)

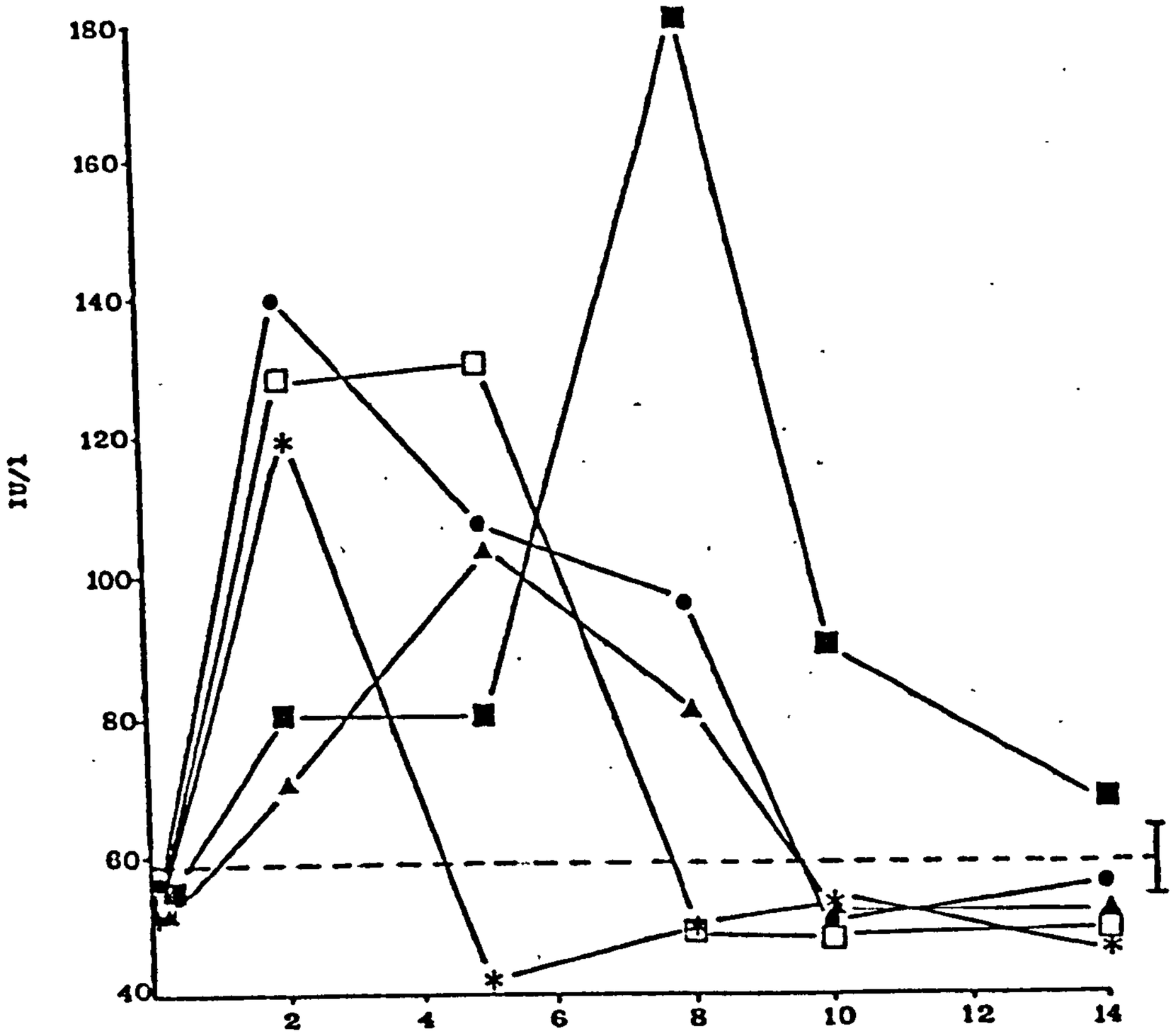


Fig. 61 Glutamine pyruvate transaminase (GPT) activity in the sera of Porton strain mice (3 wks) inoculated (a) i.c., (b) i.p. with 3 $\log_{10}$ $TCID_{50}$ of Lassa and Mopeia viruses. Points are arithmetic means of 20 mice.

● LGA391 ▲M150 □M152 ■M20410 *Z478

--------I Normal range 132

thereafter giving an irregular pattern before death. A similar initial rise in activity was noted with the non-fatal i.p. infections up to day 2 p.i., thereafter falling to normal over the following 8 days.

The isoenzymes LDH and HBDH both decreased from their normal levels (Figs. 62 and 63) after the i.c. and i.p. inoculation. In the case of LDH the serum value decreased betweeen 80-90% with each virus within the first 2 days p.i. Thereafter there was a mild recovery before death or, in the case of the non-fatal i.p. infection, no recovery was observed until 21 days p.i. which was consistent with the gross clinical observation of the animals.

Creatine kinase activity demonstrated a late response in either LGA 391 or Mopeia fatal i.c. infections. Normal levels (100 IU/l) were maintained during the first 6 days p.i. at which time they rose sharply (Fig. 64) to 350-400 IU/l. M150 and M20410 only reached levels of between 150-200 IU/l. Following this rise they returned to normal by day 8 p.i. In the case of the non-fatal i.p. infection all viruses caused an initial rise above normal reaching peak levels (200-400 IU/l) by day 2 p.i. returning to normal (100 IU/l) by day 12 p.i.

Alkaline phosphate rose from its normal level and reached maximum levels between days 4-6 after i.c. infection and decreased by day 8 p.i. to normal levels. This pattern was similar with both LGA 391 and Mopeia infections. AP activity with the non-fatal i.p. infections demonstrates (Fig. 65) an initial rise in values reaching a peak by day 2-3 p.i. and being maintained at that level until day 8 p.i. Normal values were observed from day 9 post infection. Serum creatinine and urea levels remained within normal limits.

3.6.6. Antibody levels in blood

Within the period of fatal i.c. infections no CFT or neutralising antibodies were detected. Immunofluorescent antibodies were detected during the course of the infection. With M152 and LGA 391 IFA antibodies

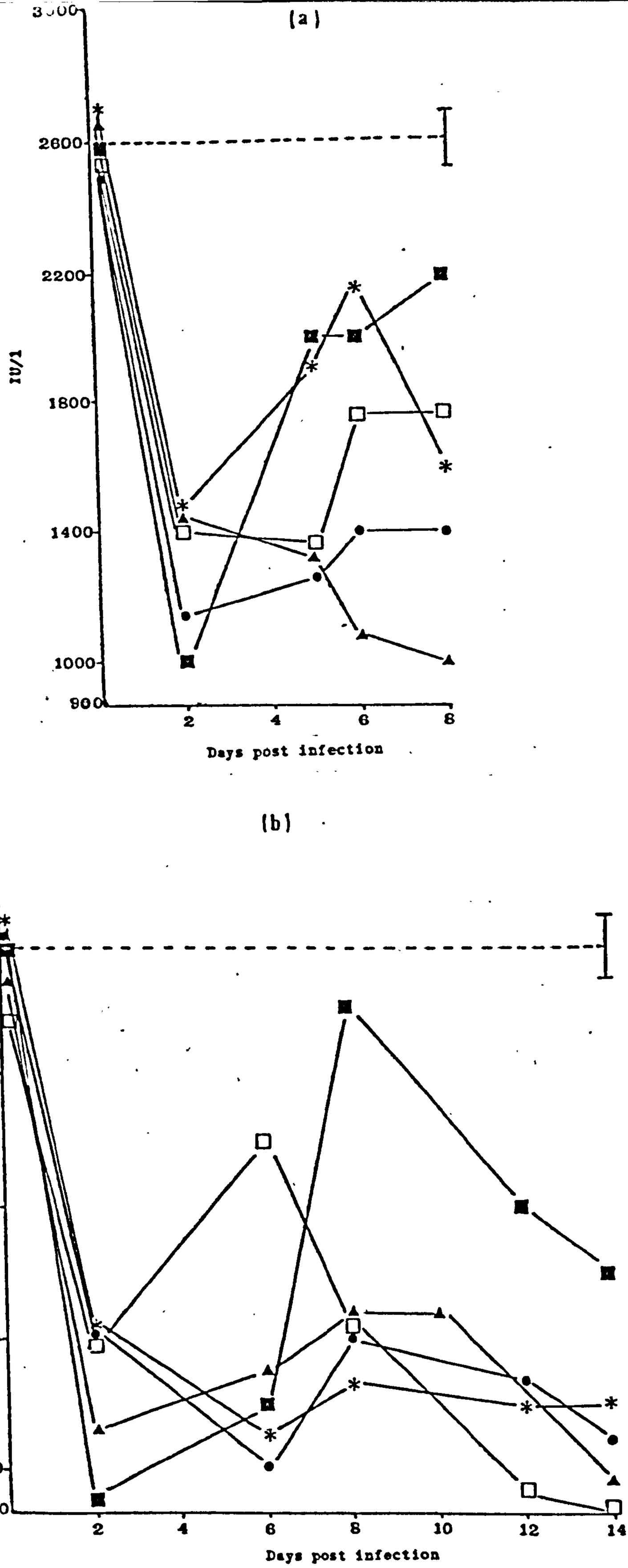


Fig. 62 Lactate dehydrogenase (LDH) activity in the sera of Porton strain mice (3 wks) inoculated i.c. (a), i.p. (b) with 3 $\log_{10}$ $TCID_{50}$ of Lassa and Mopeia viruses. Points are arithmetic means of 20 mice.

● LGA391 ▲M150 □M152 ■20410 *Z478

--------I Normal range

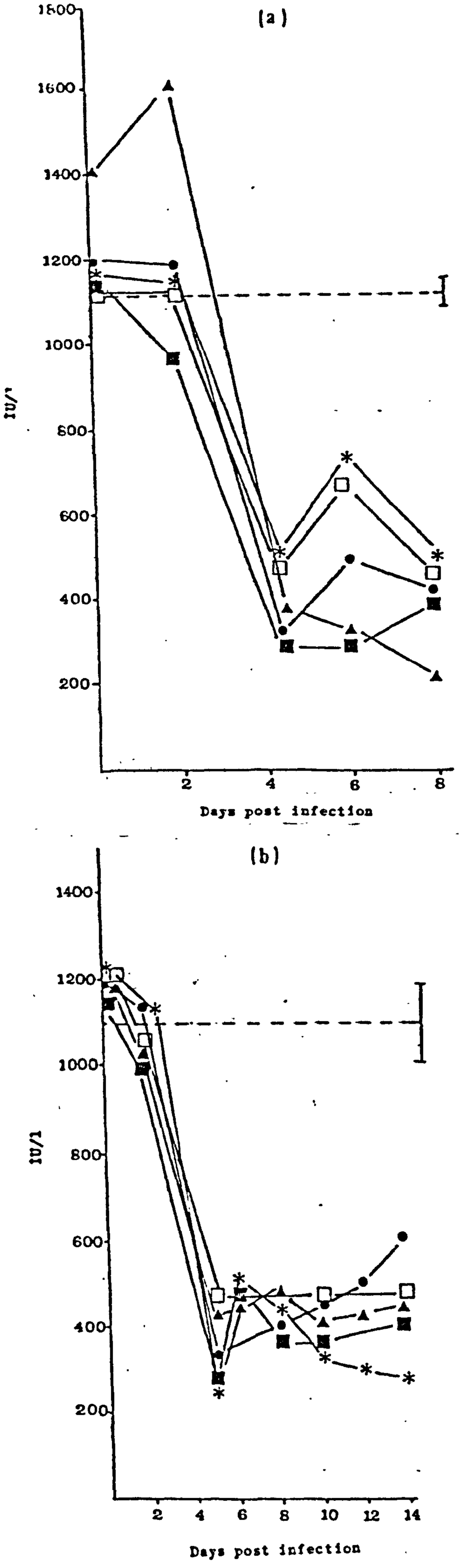
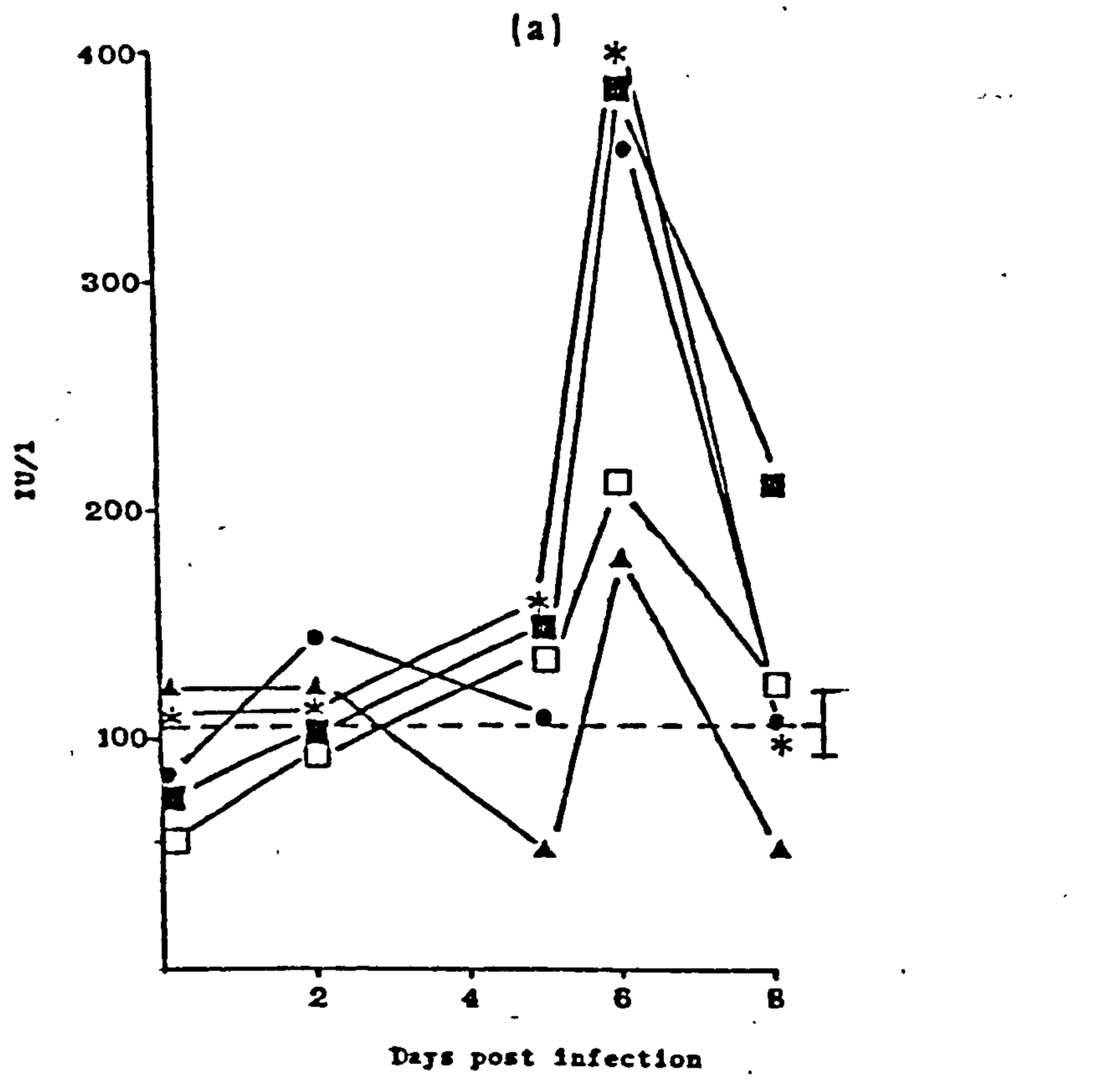


Fig. 63 α-Hydroxybutyrate dehydrogenase (α-HBDH) activity in the sera of Porton strain mice (3 wks) inoculated (a) i.c., (b) i.p. with 3 $\log_{10}$ $TCID_{50}$ of Lassa and Mopeia viruses. Points are arithmetic means of 20 mice. --------I Normal range

● LGA391 ▲M150 □M152 ■20410 *Z478



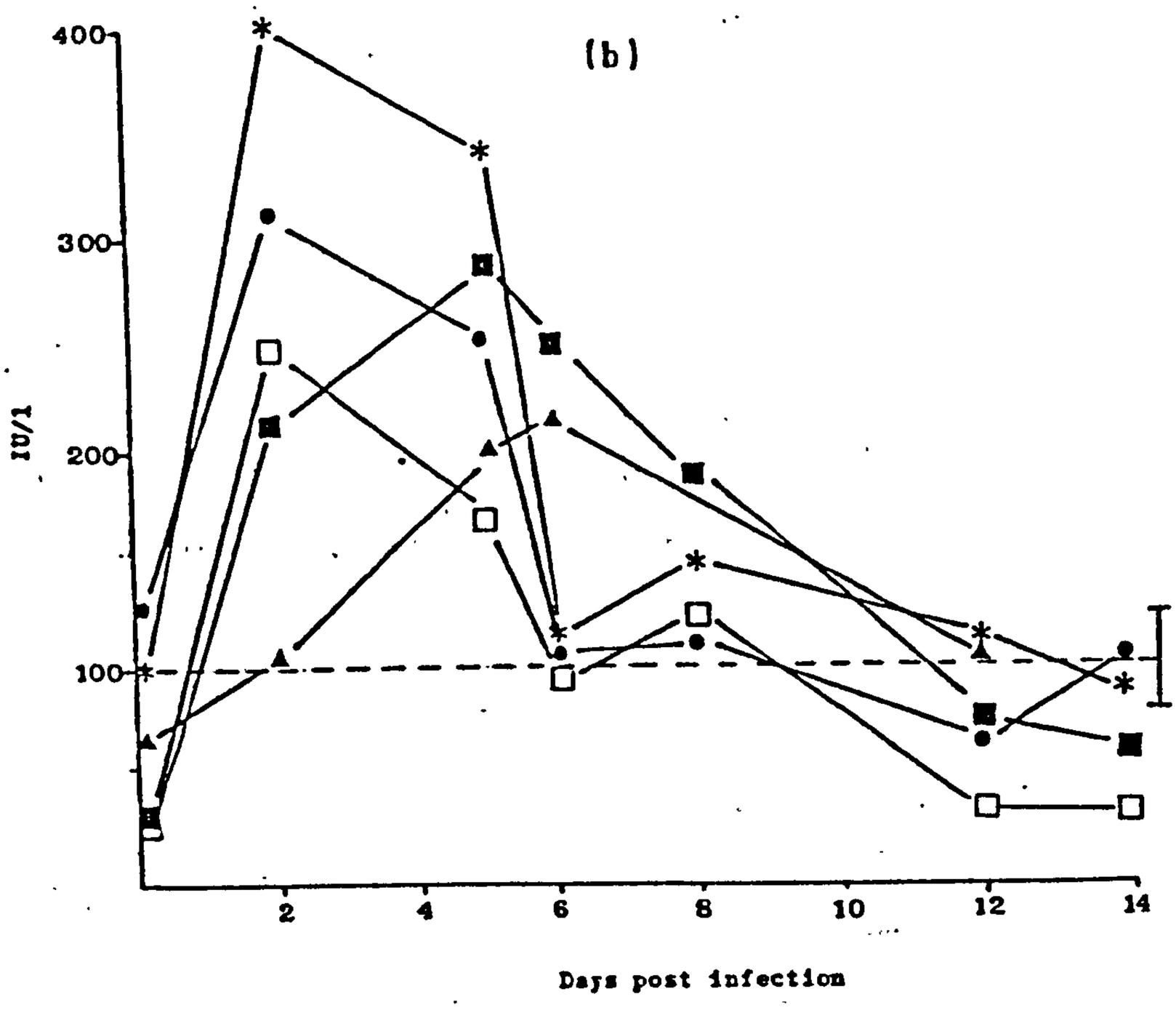


Fig. 64 Creatine kinase (CK) activity in the sera of Porton strain mice (3 wks) inoculated i.c. (a), i.p. (b) with 3 $\log_{10}$ $TCID_{50}$ of Lassa or Mopeia viruses. Points are arithmetic means of 20 mice

● LGA391 ▲M150 □M152 ■20410 ✳Z478

-------I Normal range

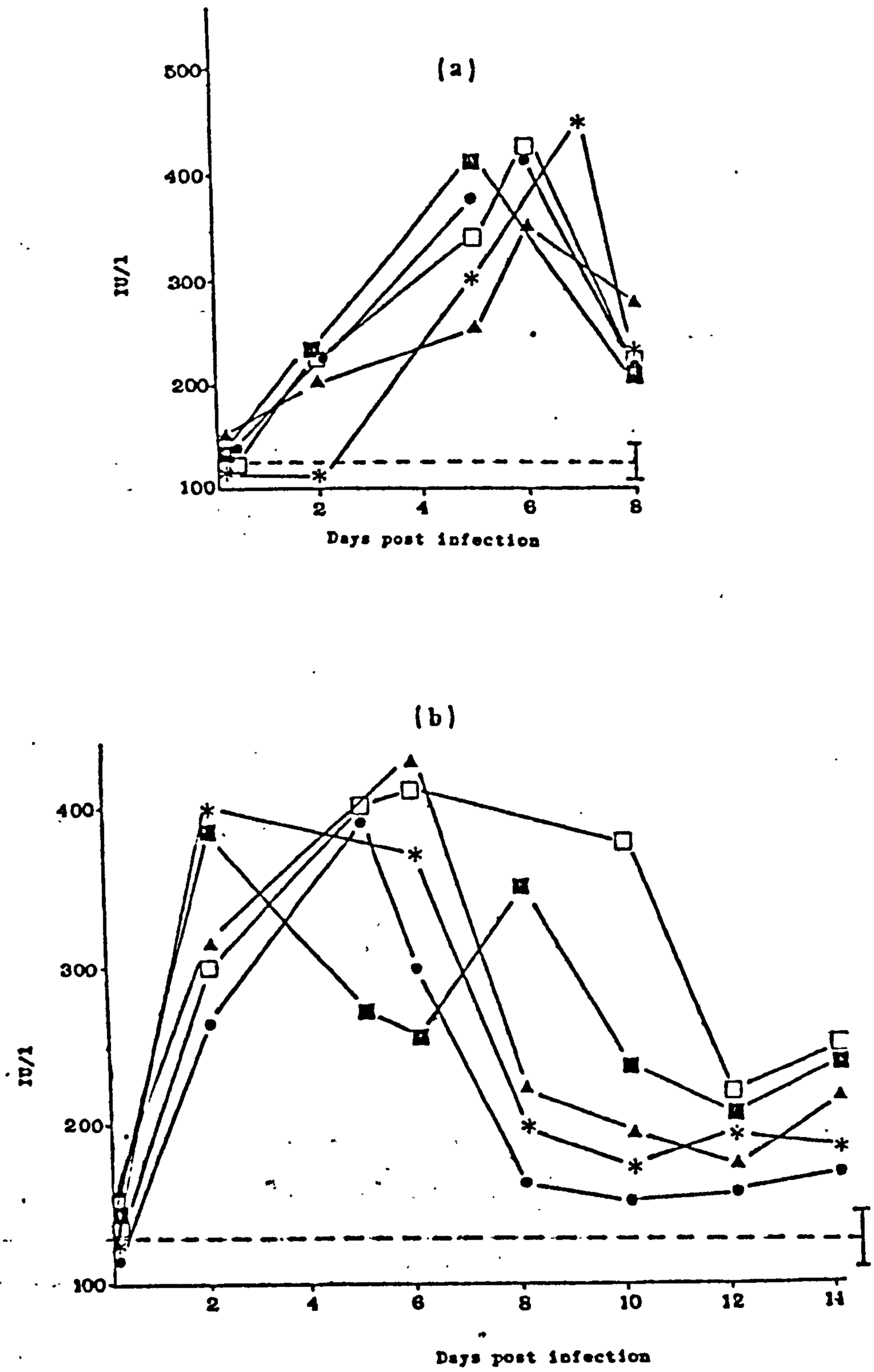


Fig. 65 Alkaline phosphatase (AP) activity in the sera of Porton strain mice (3 wks) inoculated i.e. (a), i.p. (b) with 3 $\log_{10}$ $TCID_{50}$ of Lassa or Mopeia viruses.· Points are arithmetic means of 20 mice.

● LGA391 ▲M150 □M152 ■20410 *Z478

-------I Normal range

were first detected on day 2 p.i. and reached maximum levels on day 9 p.i. of 1:128 and 1:512 respectively. Mopeia viruses 20410, 150 and 478 initiated a detectable IFA response on day 4 p.i. reaching between 1:512-1:4096 (Figs. 57 and 58).

With the non-fatal i.p. initiated infection IFA antibodies were detected between day 2 and 4 p.i. (Figs. 57 and 58) reaching maximum levels (1:2048-1:4096) between day 10 and 12 p.i.

Homologous neutralization antibodies were not observed during the 21-day study with each virus. In response to Mopeia infection, the production of anti-Lassa neutralization antibodies was not observed.

3.7. TRANSMISSION OF LASSA AND MOPEIA VIRUSES TO DUNKIN-HARTLEY GUINEA PIGS

Tissue culture pools of stock virus were inoculated into male and female guinea pigs weighing 350-500 g.

3.7.1. Susceptibility

The susceptibility of guinea pigs to different routes of inoculation are shown in Table 37. No difference was observed between males and females.

LGA391 virus killed 70% of the guinea pigs inoculated by i.p., s.c. and i.m. routes with 3 $\log_{10}TCID_{50}$. By comparison, M152 was the only Mopeia virus that demonstrated any lethal effect on guinea pigs (Table 37) when inoculated with >3 $\log_{10}TCID_{50}$. All guinea pigs surviving infection (Table 37) seroconverted by the IFA tests and resisted further challenge with the homologous virus, suggesting that inapparent infections had occurred.

TABLE 37. INFECTIVITY AND LETHALITY OF LGA391 AND MOPEIA VIRUS STRAINS IN GUINEA PIGS (DUNKIN-HARTLEY) INOCULATED I.P., S.C. OR I.M.

Virus	Inoculum $TCID_{50}$/0.1 ml	No dead/No treated (%)	Mean day of death (range)	Sero converted	Resisted a challenge
LGA391	$>10^5$	22/30(73.3)	15.5(11-17)	30/30	30/30
	10^3	21/30(70)	17.1(16-19)	30/30	30/30
	10^1	0/30		30/30	30/30
M152	$>10^5$	12/30(40)	16(10-16)	30/30	30/30
	10^3	9/30(30)	18(15-20)	30/30	30/30
	10^1	0/30		30/30	30/30
M150	$>10^5$	0/30	-	30/30	30/30
	10^3	0/30	-	30/30	30/30
	10^1	0/30	-	30/30	30/30
M20410	$>10^5$	0/30	-	30/30	30/30
	10^3	0/30	-	30/30	30/30
	10^1	0/30	-	30/30	30/30
Z478	$>10^5$	0/30	-	30/30	30/30
	10^3	0/30	-	30/30	30/30
	10^1	0/30	-	30/30	30/30

a. Lived 45 days or longer after i.p. or s.c. inoculation of 3 $\log_{10}TCID_{50}$ with homologous virus.

3.7.2. Clinical observations

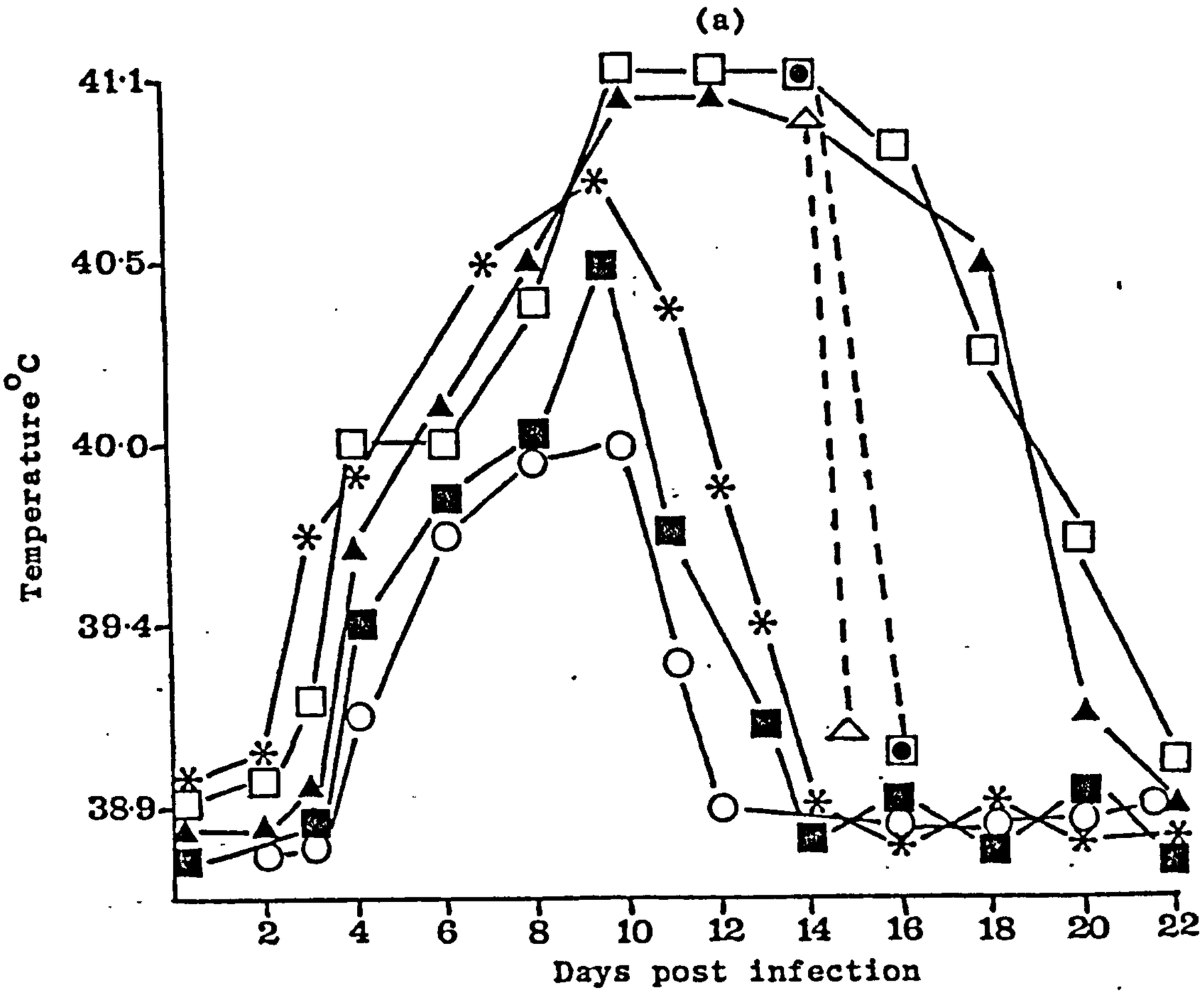
Guinea pigs became febrile after infection with LGA 391 or Mopeia viruses (Fig. 66a and b). The severity of the pyrexia was dependent on the infective dose and strain of virus. The route of infection, s.c., i.p. or i.m., did not alter the outcome of the disease.

Infecting guinea pigs with LGA391 or M152 at a level between 2-6 $\log_{10}TCID_{50}$ demonstrated two pyrexial responses (Fig. 66a and b), one following a course of infection leading to recovery, the other being lethal.

Following a two day incubation period guinea pigs became febrile, gradually reaching a peak of of 40.9 - 41.1°C on day 10 p.i. persisting until day 16 p.i. Normal temperature levels (38.2 - 38.9°C) were recorded by day 22 p.i. in guinea pigs which recovered. As a result of a lethal infection, high temperatures persisted from day 10 to day 15. On day 16 there was a marked fall in temperature to 39.0°C - 39.2°C which consistently signalled the animals' death. A lethal infection did not occur with LGA391 or M152 when the infective dose was <1 $\log_{10}TCID_{50}$ but a febrile reaction was observed similar to those produced with higher infective doses (Fig. 66a). Peak pyrexia (41.1°C) was observed at day 10, remained until day 15 and fell to normal levels by day 20.

A mild febrile illness also occurred when guinea pigs were inoculated with M150, M20410 and Z478 viruses but with no lethal outcome. The temperature responses were more dependent upon the dose of infection. At infective doses ≥6 $\log_{10}TCID_{50}$, there was a similar 2 day incubation period. Pyrexia developed after the second day and peaked on day 10 p.i., with M150, Z478 and M20410. No plateau was observed as with LGA391 or M152, and the temperature gradually returned to normal by day 14 in each case (Figs.66a and b). Using an infective dose of 4 $\log_{10}TCID_{50}$, a similar pyrexial pattern was observed (Fig. 66b). Temperature peaks were lower but still occurred on day 10 p.i. The fall to normal temperature levels took 4 days longer. Observations on guinea pigs receiving 2-3 $\log_{10}TCID_{50}$ demonstrated a marked depression in febrile response, peak temperatures (39.6°C) occurring between day 6 -10 p.i., falling to normal by day 16 p.i.

A similar picture was observed when guinea pigs were inoculated with 1 $\log_{10}TCID_{50}$ although there were small alterations in responses. Z478 infection caused a rapid onset of febrile illness peaking at day 6 at 40.2°C, returning to normal by day 10 p.i. Guinea pigs inoculated with M150 and M20410 gave rise to a mild febrile illness peaking at day 6, returning to normal by day 11-12 p.i.



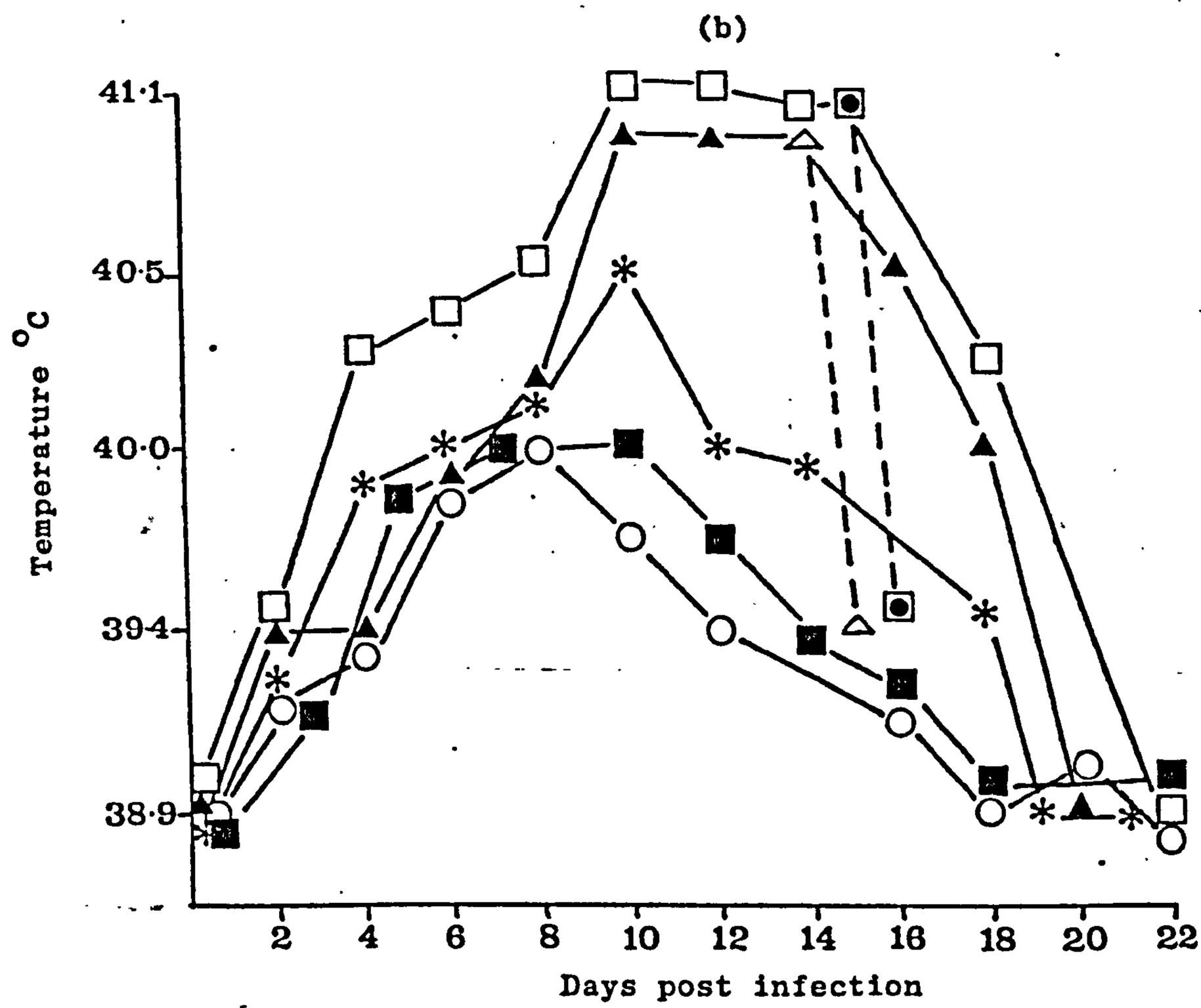


Fig. 66. Graphs demonstrating the temperature course in guinea pigs inoculated i.p. with (a) 6 $\log_{10}$TCID$_{50}$ (b) 4 $\log_{10}$TCID$_{50}$

▲LGA 391 □M152 ✱M150 ○M20410 ■Z478

▲----▲ and ◉----◉ lethal course of infection

Each point represents the arithmetic mean of 30 animals.

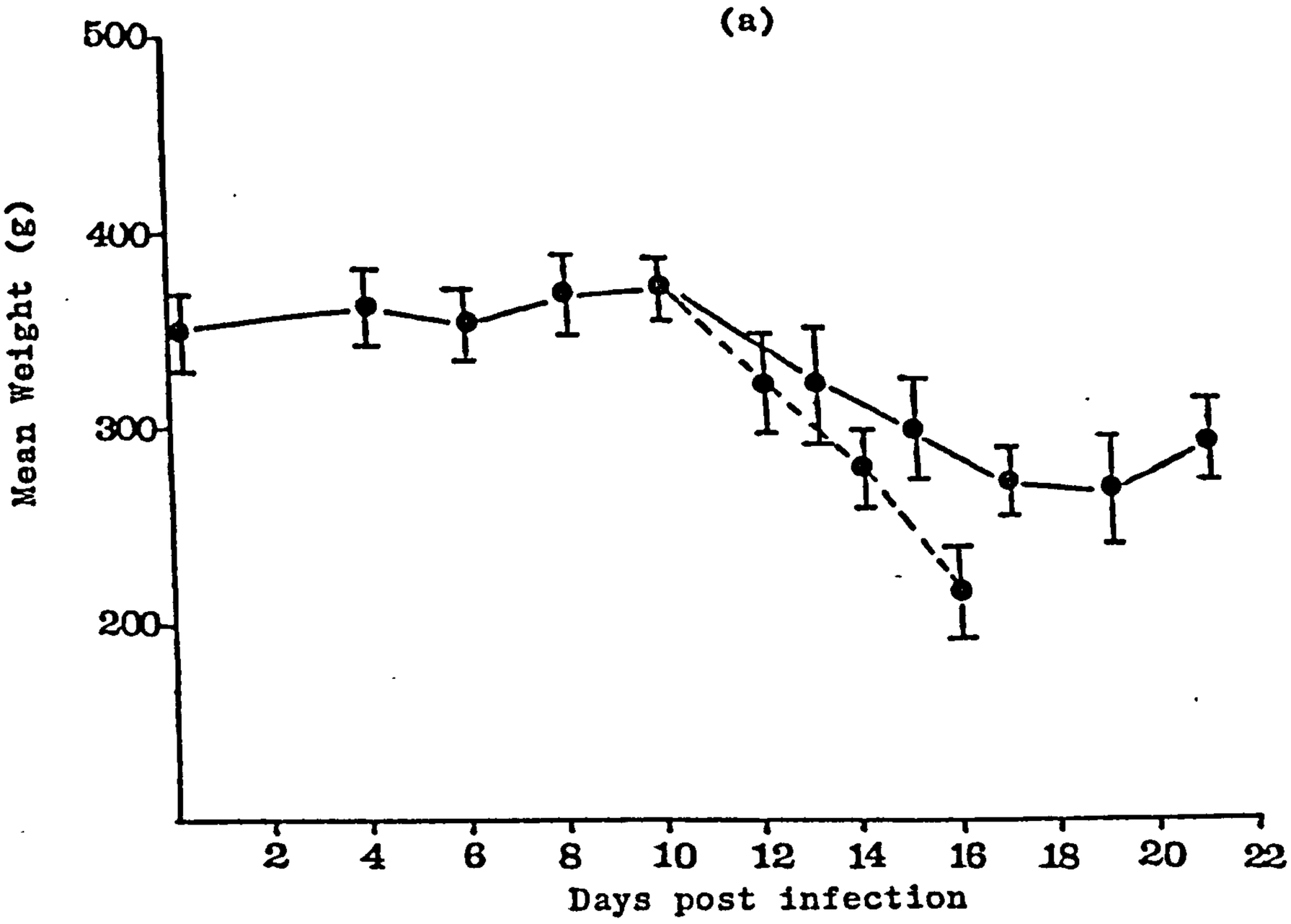


Fig.67. Graph demonstrates the mean weight of guinea pigs inoculated with 3 $\log_{10}$ $TCID_{50}$ ml of either LGA 391 or M152

●——● Non-lethal infection ●-----● lethal infection

Each point represents the arithmetic mean of 20 guinea pigs.

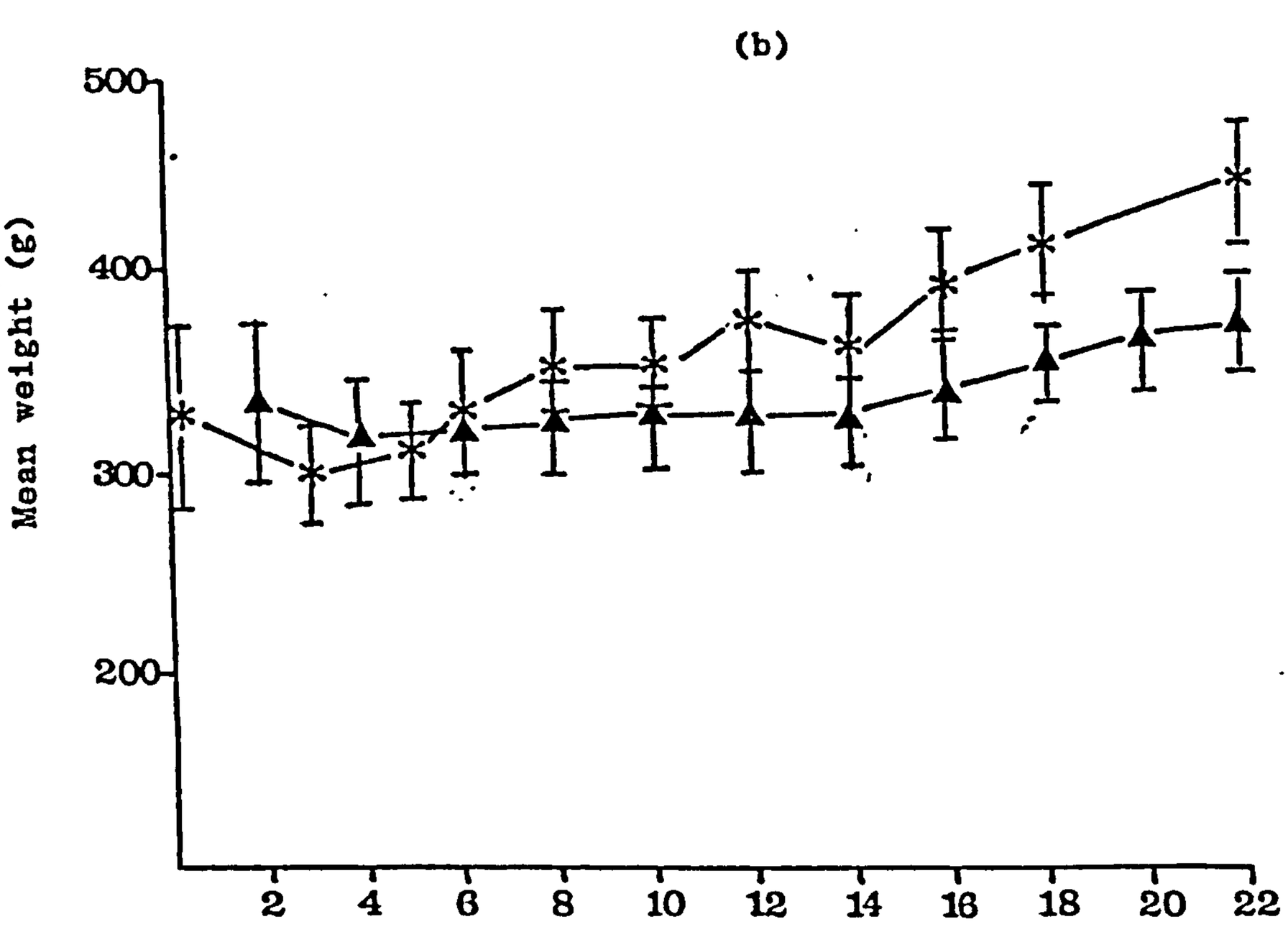


Fig.68. Graph demonstrates the mean weight of guinea pigs inoculated with 3 $\log_{10}TCID_{50}$ of Mopeia virus (M150, M20410, Z478) and a normal control.

▲ Mopeia viruses Control *

Each point represents the arithmetic mean of 20 guinea pigs.

During the course of all the infections the guinea pigs were inactive during the febrile phase of illness and reduced their intake of food and water, which affected their weight and general condition. Noticeably, those guinea pigs inoculated with LGA391 and M152 were the most severely affected, even those which survived. The animals did not put on any weight during the first 10 days p.i., after which there was a gradual weight reduction to 190-240g until death. Those animals which recovered began gaining weight from day 22 p.i. (Fig. 67). The weight of guinea pigs inoculated with M150, M20410 and Z478 did not increase from average (300-375g) during the first 14 days p.i. All started to gain weight after 14 days p.i. (Fig. 68) but not as rapidly as the controls.

3.7.3. Virus levels in blood and tissue

To characterize the LGA 391 and Mopeia virus infections of guinea pigs, comparative virus titres were made among groups of 20 guinea pigs inoculated with 0.1 ml 3 $\log_{10}$ $TCID_{50}$ and bled daily until day 21 or until they died (Figs. 69 and 70).

In the case of LGA391 serum viraemia reached peak levels during the first 8-10 days p.i. both in lethal and surviving guinea pigs. In lethally infected guinea pigs, the viraemia reached >4 $\log_{10}$ $TCID_{50}$/ml and persisted above 2.5 $\log_{10}$ $TCID_{50}$/ml until death. In guinea pigs which survived, peak viraemia of 3.5 $\log_{10}$ $TCID_{50}$/ml was achieved by day 8 p.i., and persisted to day 10 p.i., thereafter declining to undetectable levels by day 18 p.i. (Fig. 69). Similar viraemic patterns were observed in guinea pigs infected with M152, except that the percentage lethalities were considerably less than with LGA391 (Table 37).

Viraemia in guinea pigs infected with the Mopeia viruses demonstrated little or no virus ($<10^1 - 10^{1.5}$ $TCID_{50}$/ml) by day 4-5 p.i. returning to undetectable limits during the remainder of the 21 day study (Figs. 69 and 70). The demonstration of serum viraemia proved inconsistent in guinea pigs infected with any of the Mopeia viruses.

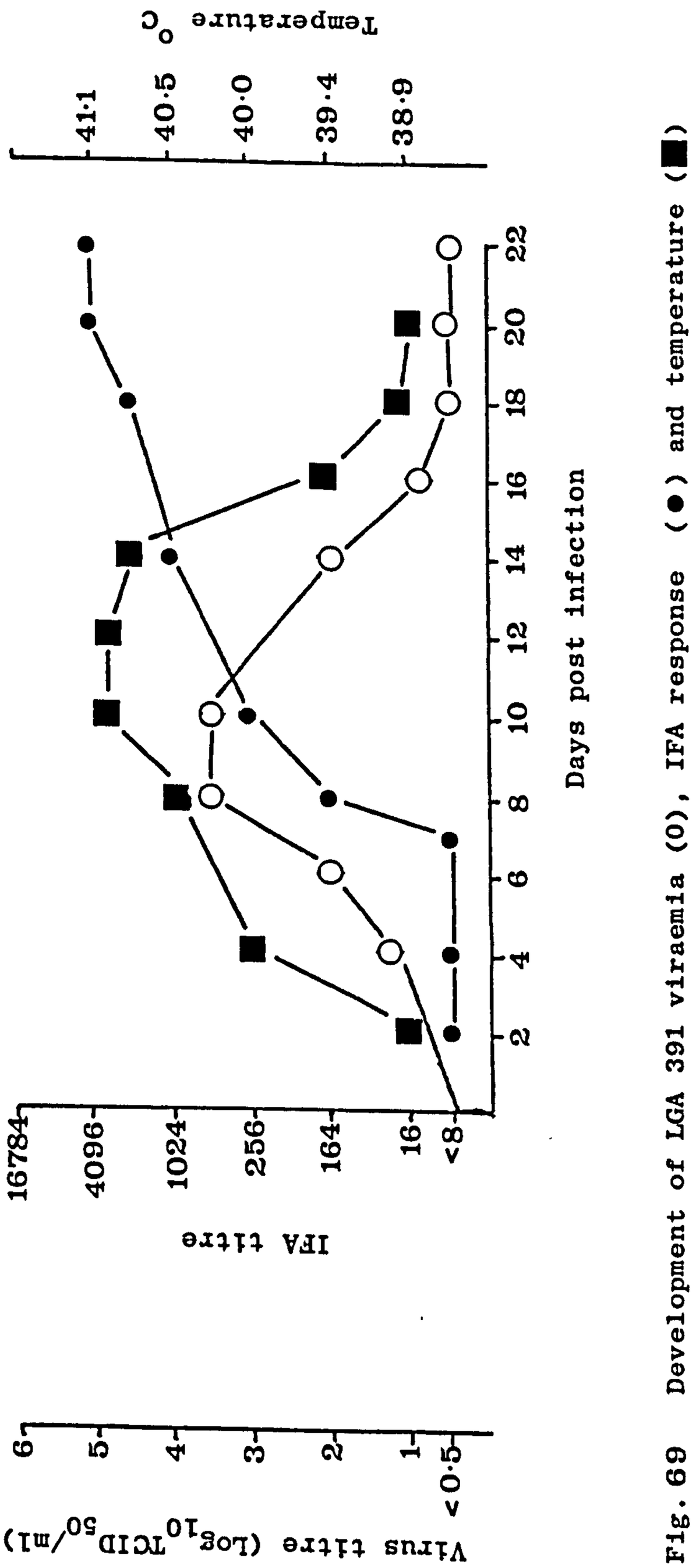


Fig. 69 Development of LGA 391 viraemia (O), IFA response (●) and temperature (■) in surviving guinea pig inoculated i.p. with 3 log_{10} $TCID_{50}$ of virus.

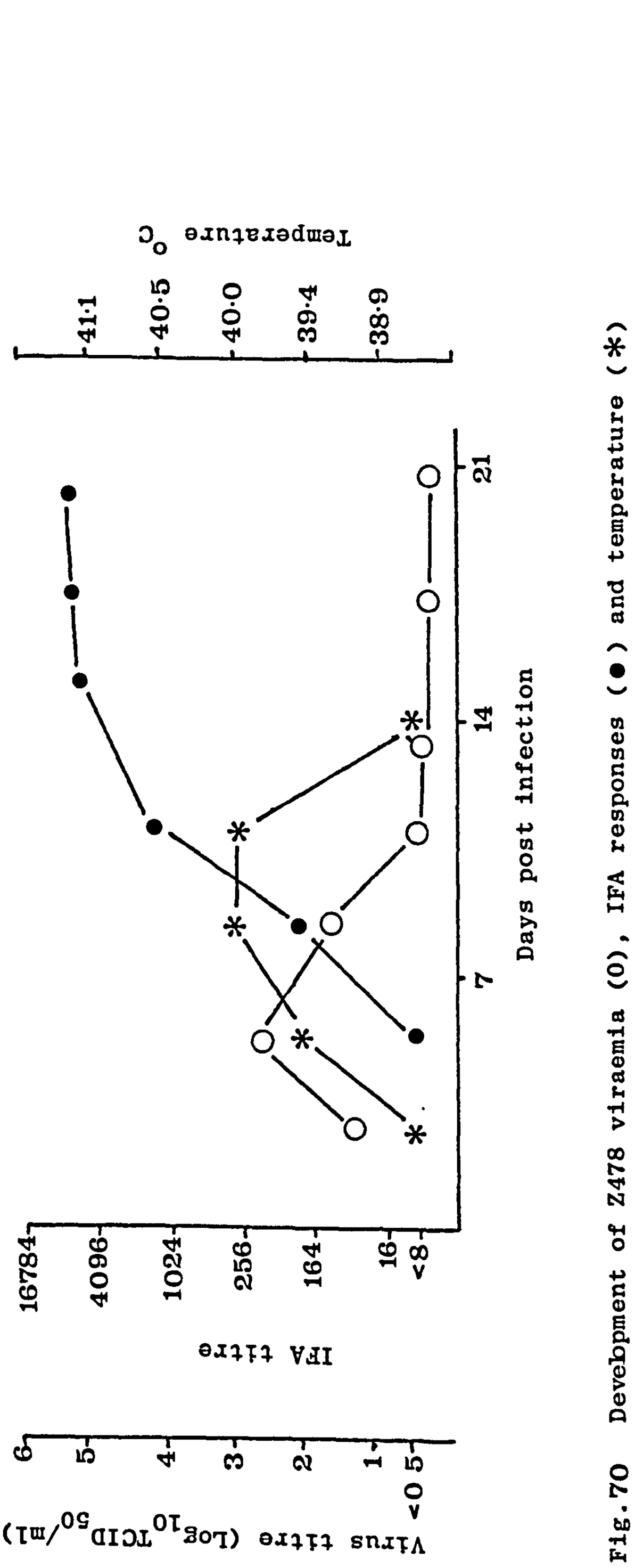


Fig.70 Development of Z478 viraemia (O), IFA responses (●) and temperature (*) in guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$ of virus.

To obtain additional insight into the tissue tropisms and histological lesions produced by LGA391 and Mopeia viruses, various organs were removed from the guinea pigs. In the case of LGA391 infected animals, this was done when the animals were found to be moribund (day 14-18). Tissues were removed from M150, M20410 and Z478 guinea pigs sacrificed through the course of infection. Infectivity data for the various tissues suggested that LGA391 replicated in all of the extraneural tissues examined but not in the brain. High titres of LGA391 were found early (day 10-14) in the mesenteric lymph node, spleen and lung. In the moribund animals examined 14-18 days after infection, the highest titres of virus were recovered from the lung, liver, mesenteric lymph node and spleen, followed by the adrenals, kidney, heart and salivary gland (Fig. 71). Virus recovery from the brain was too low a titre to suggest blood-borne virus.

Mopeia viruses were only recovered at low levels from the liver, spleen and mesenteric lymph nodes even in those guinea pigs demonstrating low serum viraemia (Fig. 71). Virus recovery was variable and only made during the 4-6 day period post-infection; thereafter no virus was detected. The isolation of Mopeia viruses from some guinea pigs was never achieved throughout infection even though IF antibody was detected. This pattern was characteristic of M150, M20410 and Z478. The M152 virus infected guinea pigs that survived, demonstrated the same pattern as the other Mopeia virus infections. However in those guinea pigs displaying a lethal infection, tissue virus levels mimicked LGA391. Virus was detected primarily in the spleen, liver, mesenteric lymph node and adrenals at levels between 2-4 $\log_{10}$ $TCID_{50}$/ml/g of tissue. Other tissue isolates could be attributed to blood-borne virus.

3.7.4. Antibody levels in blood

Humoral antibodies to LGA 391 and Mopeia viruses were measured in guinea pigs inoculated with 3 $\log_{10}$ TCID50 by the i.p.route.

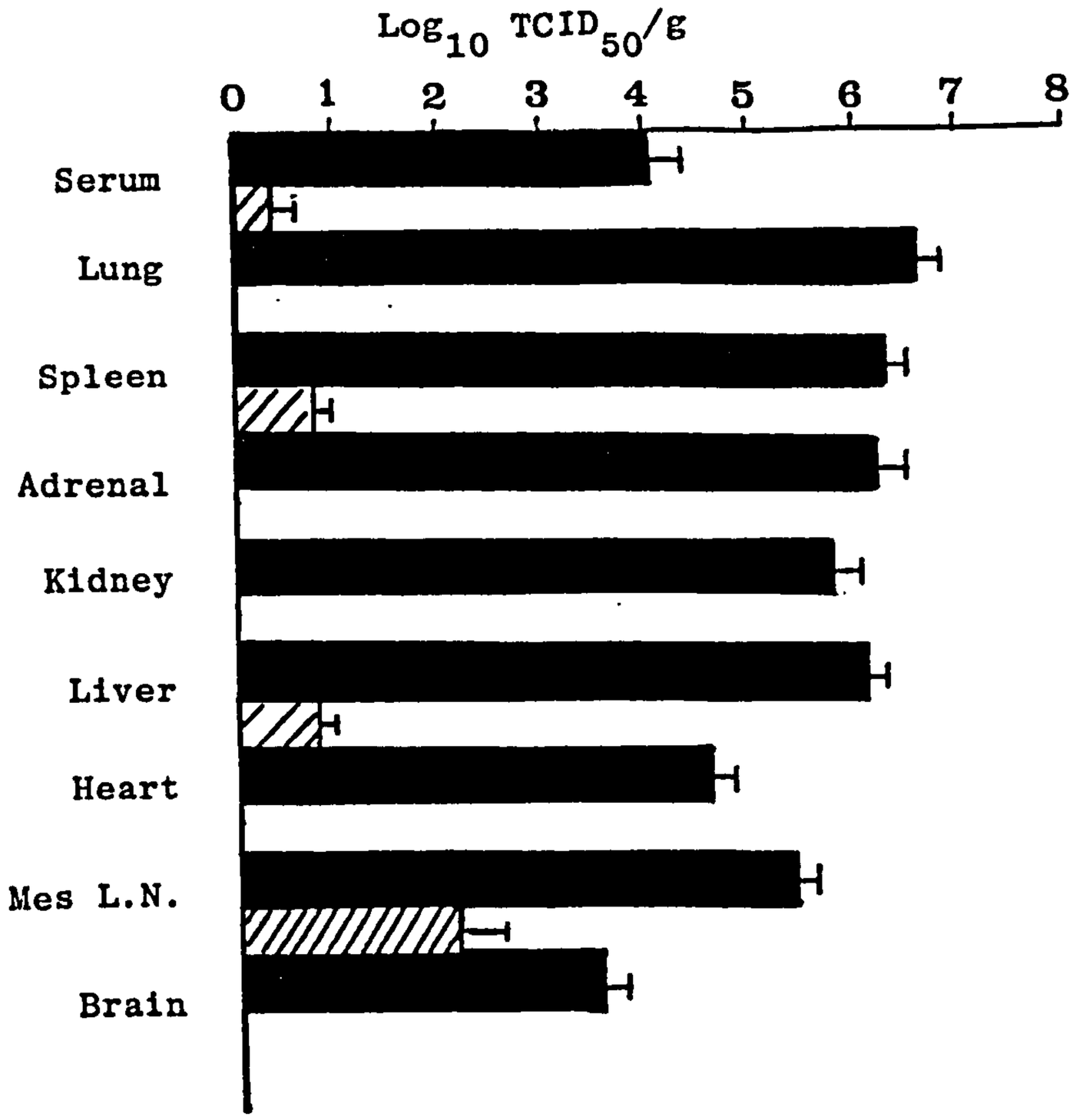


Fig.71 Comparative concentrations of LGA 391 (■) virus and Mopeia viruses (▨) recovered from serum and tissues of guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$/ml.

Titres are geometric means (± standard error) of (a) 10 moribund guinea pigs of LGA 391 (day 14-18), and (b) 10 guinea pigs of M150 at the height of serum viraemia (day 4-6) as no virus was detected before or after this time.

Immunofluorescent antibody (I.F.A.) were first detected between day 6 and 8. All subsequent development of I.F. antibodies followed a similar pattern as demonstrated in Table 38. The IFA titres reached 1 : 64 or more by day 10 i.p. and 1 : 512 to 1 : 2048 by day 16 p.i. with all the strains of Lassa and Mopeia virus.

TABLE 38. APPEARANCE OF IMMUNOFLUORESCENT ANTIBODIES IN GUINEA PIGS INFECTED WITH 3 LOG_{10} $TCID_{50}$ OF LGA 391 AND MOPEIA VIRUS

	VIRUSES				
Days p.i.	LGA391	M152	M150	M20410	Z478
4	8	8	8	8	8
6	8	16	8	8	8
8	64	256	16	16	8
10	256	ND	64	64	128
12	512	512	256	ND	1024
14	512	ND	ND	1024	
16	ND	1024	1024	1024	2048
18	2048	ND	512	1024	4196
20	4196	2048	4196	2048	2048
22	8392	ND	8392	2048	2048
24	ND	4196	8392	2048	2048
26	ND	4196	8392	2048	2048
28	4196	4196	8392	2048	2048
30	4196	4196	8392	2048	2048

The cross-immunofluorescent capabilities of hyperimmune guinea pig sera are dealt with in Section 3.4.

The study of neutralization (N) antibody did not show the same consistency of production as with IFA. N-antibody titres were first detected around day 60 p.i. in guinea pigs inoculated with 3 log_{10} $TCID_{50}$ (LGA 391) and increased with time for 140 days, the last time tested (Table 39). N-antibodies were first detected on day 15 p.i. in animals inoculated with all the Mopeia viruses including M152. The N-antibody titres began to rise between day 25 and 30 p.i. and increased with time for 80-90 days,

remaining at their peak till 140 days p.i. No anti-Lassa neutralising antibodies were found in any Mopeia infected guinea pigs during the 140 days of study.

TABLE 39. THE APPEARANCE OF HOMOLOGOUS NEUTRALIZING ANTIBODIES IN GUINEA PIGS INOCULATED WITH LGA 391 OR MOPEIA VIRUSES

	NEUTRALIZATION INDEX									
	Days									
Virus	10	15	20	25	30	35	60	90	120	140
LGA391	0	0	0	0	0	0	2.1	3.2	3.5	3.5
M152	0	0	0	0	0	0	1.0	2.8	3.0	3.2
M150	0	0	0	0	0.5	ND	ND	2.0	3.2	3.2
M20410	0	0	ND	0.6	1.0	ND	1.2	2.0	3.2	3.2
Z478	0	0	ND	0.8	1.2	ND	1.7	2.7	2.7	2.7

3.7.5. Clinical Biochemistry

To evaluate the significance of altered plasma enzymes and substrate values as a result of arenavirus infection, normal values for each parameter were first calculated for the Dunkin-Hartley Guinea Pig model (Table 40).

TABLE 40. NORMAL VALUES OF SERUM ENZYMES AND SUBSTRATES IN DUNKIN-HARTLEY GUINEA PIGS

Enzyme/Substrate	Normal value*	±SE	Units
GOT	125.60	40.4	IU/l
GPT	73.96	12.6	"
LDH	361.34	33.1	"
α-HBDH	140.3	23.1	"
CK	448	63.4	"
γ-GT	23.32	7.1	"
AP	248.8	33.4	"
Creatine	0.3	0.1	mg/100ml
Urea	55.08	2.2	"
Total protein	5.30	0.2	"
Triglycerides	100.75	11.1	"
Cholesterol	74.76	10.4	"

*Each value represents analysis of 30 samples.

The alterations in enzyme levels in guinea pig serum reflected the degree of clinical illness caused by LGA 391 and Mopeia viral infections (Figs. 72-80). These can be divided into three categories and can be attributed to the various strains arenavirus.

Degree of change	Virus
Highly significant	LGA391, M152
Moderately significant	LGA391, M152
Insignificant	M150, M20410, Z478

The GOT, GPT, LDH, α-HBDH, AP and CK serum enzymes all demonstrate significant alterations to LGA391 and M152 infections, resulting in a fatal or non-fatal infection and each of the above enzymes reflected the ultimate course of the disease (Table 41).

In both fatal and non-fatal infections enzyme levels start to rise between days 2 and 3 p.i. Maximum levels were reached 5 days p.i. in fatal infections (Figs. 72, 74, 76, 78, 80a). A more gradual course was observed with non-lethal infections, peak levels being reached between days 6 and 9 (Table 41). As will be noted in both table and figures, enzyme levels reached higher levels in lethal infections and were maintained throughout the remainder of the infections. Enzyme levels returned to normal in surviving guinea pigs between days 10 and 18 p.i. dependent on enzyme (see Table 41 and Figs. 72-80). The CK value in fatal cases increased 10-fold. The peak value was achieved between 7 and 8 days p.i. During the same period in non-fatal LGA 391 and M152 infections the CK value doubled, returning to normal by day 10 p.i. The γGT, Creatine, Urea, Total Protein, Triglycerides and Cholesterol level did not alter significantly from normal.

Only the CK values demonstrated any noticeable increase with M20410, M150 and Z478 viral infections between day 7 and 8 p.i. (Fig. 80b),

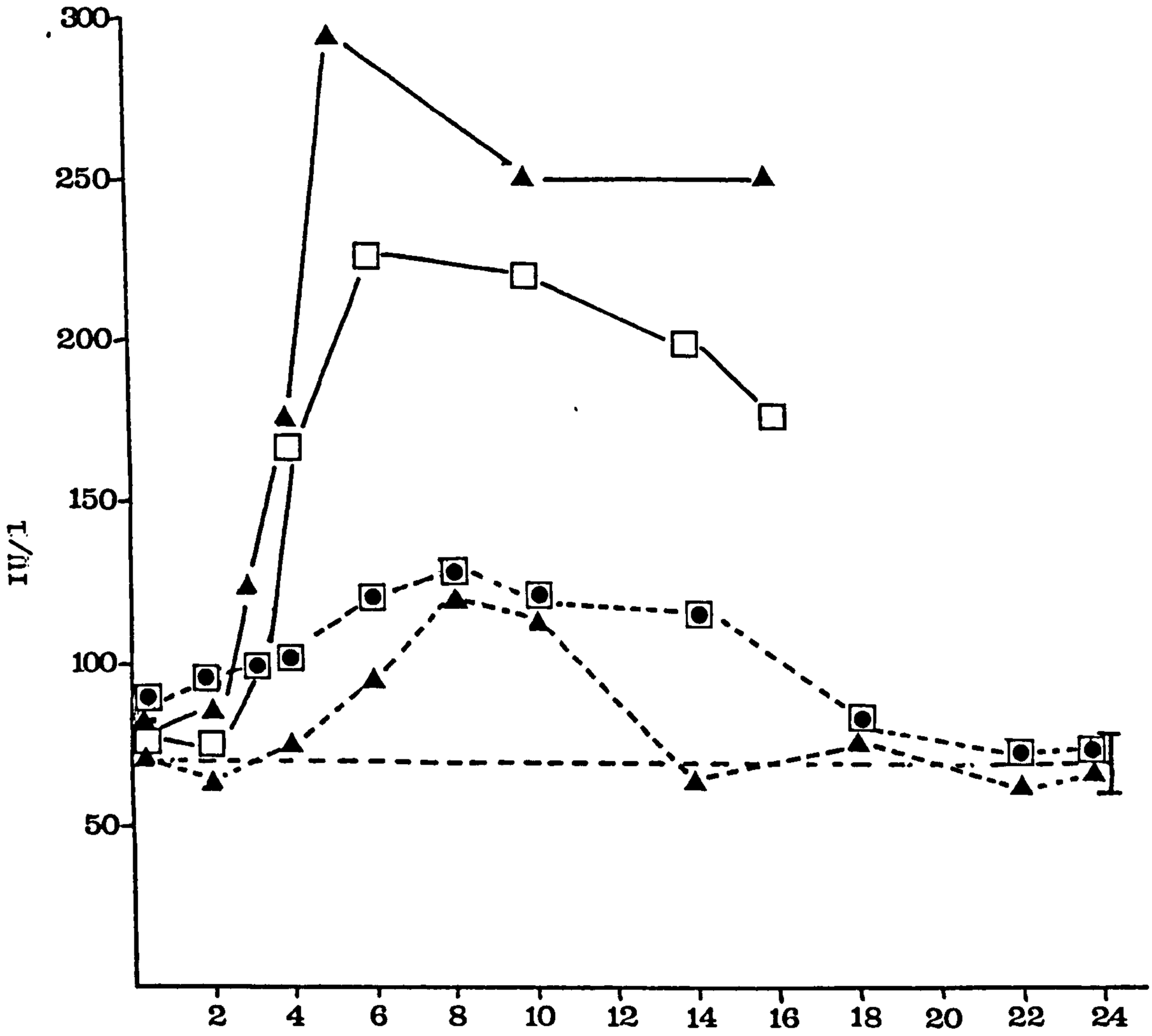


Fig.72 GOT activity in the sera of guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$ of LGA 391 and M152 viruses giving a lethal (——) and non-lethal (----) infection.

□——□M152 ▲——▲LGA 391

◉----◉M152 ▲----▲LGA 391

------I Normal values

Each point represents the arithmetic mean of 25 guinea pigs.

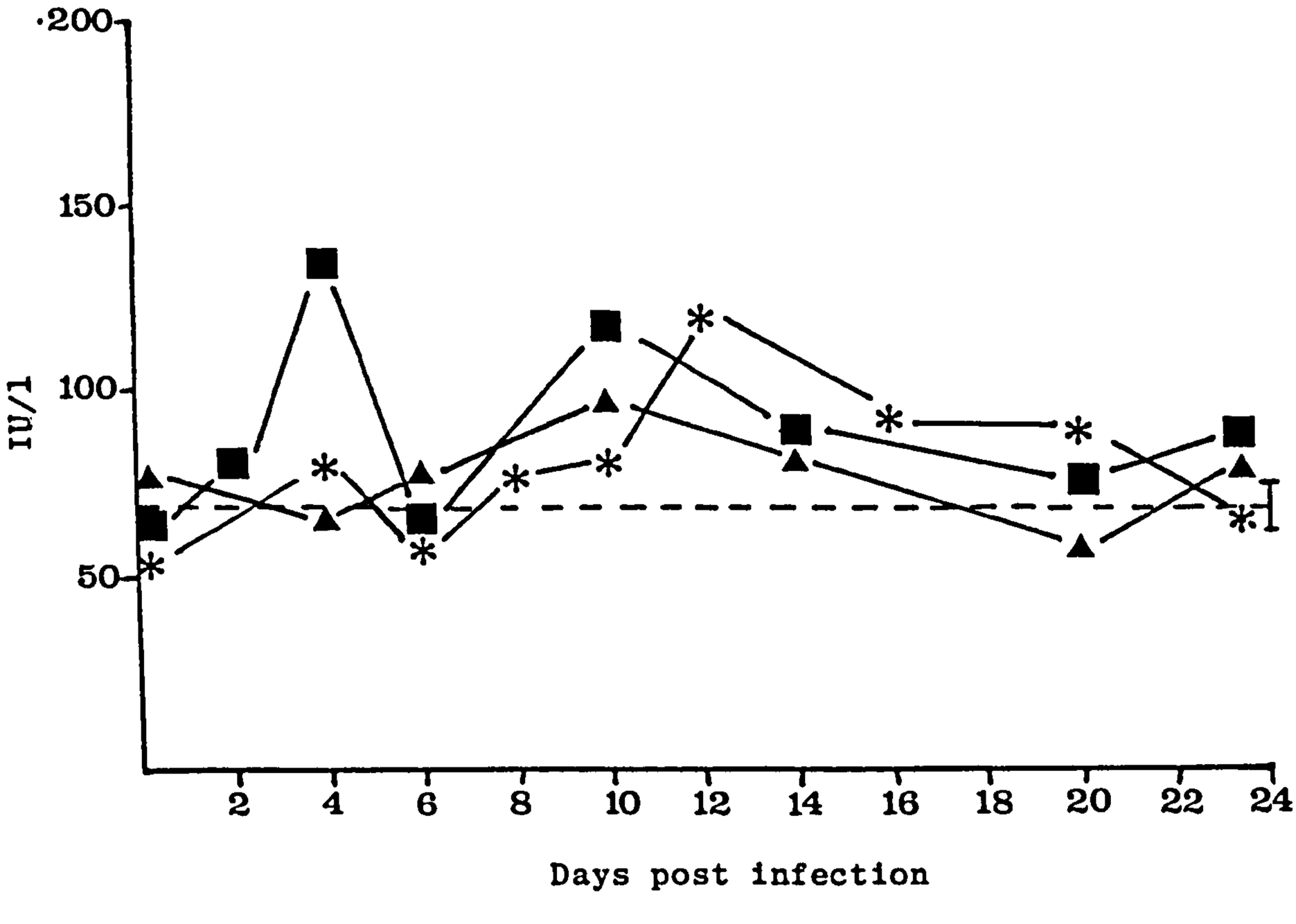


Fig.73 GOT activity in the sera of guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$ of Mopeia viruses.

▲M150 ■M20410 *Z478

------I Normal values

Each point represents the arithmetic mean of 25 guinea pigs.

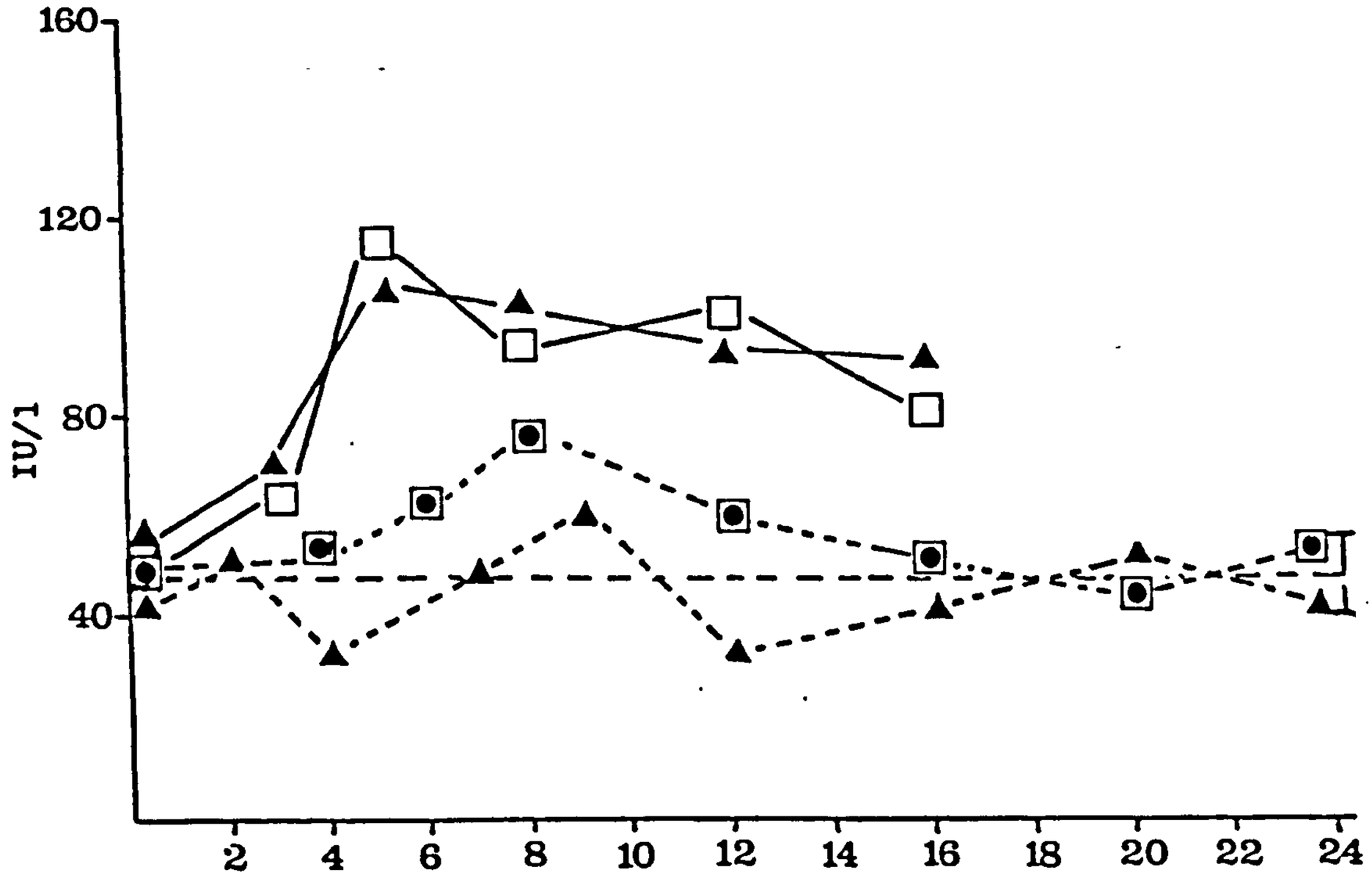


Fig.74 GPT activity in the sera of guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$ of LGA 391 and M152 viruses giving a lethal (——) and non-lethal (----) infection.

□——□ M152 ▲——▲ LGA 391

◉---◉ M152 ▲----▲ LGA 391

------I Normal values

Each point represents the arithmetic mean of 30 guinea pigs.

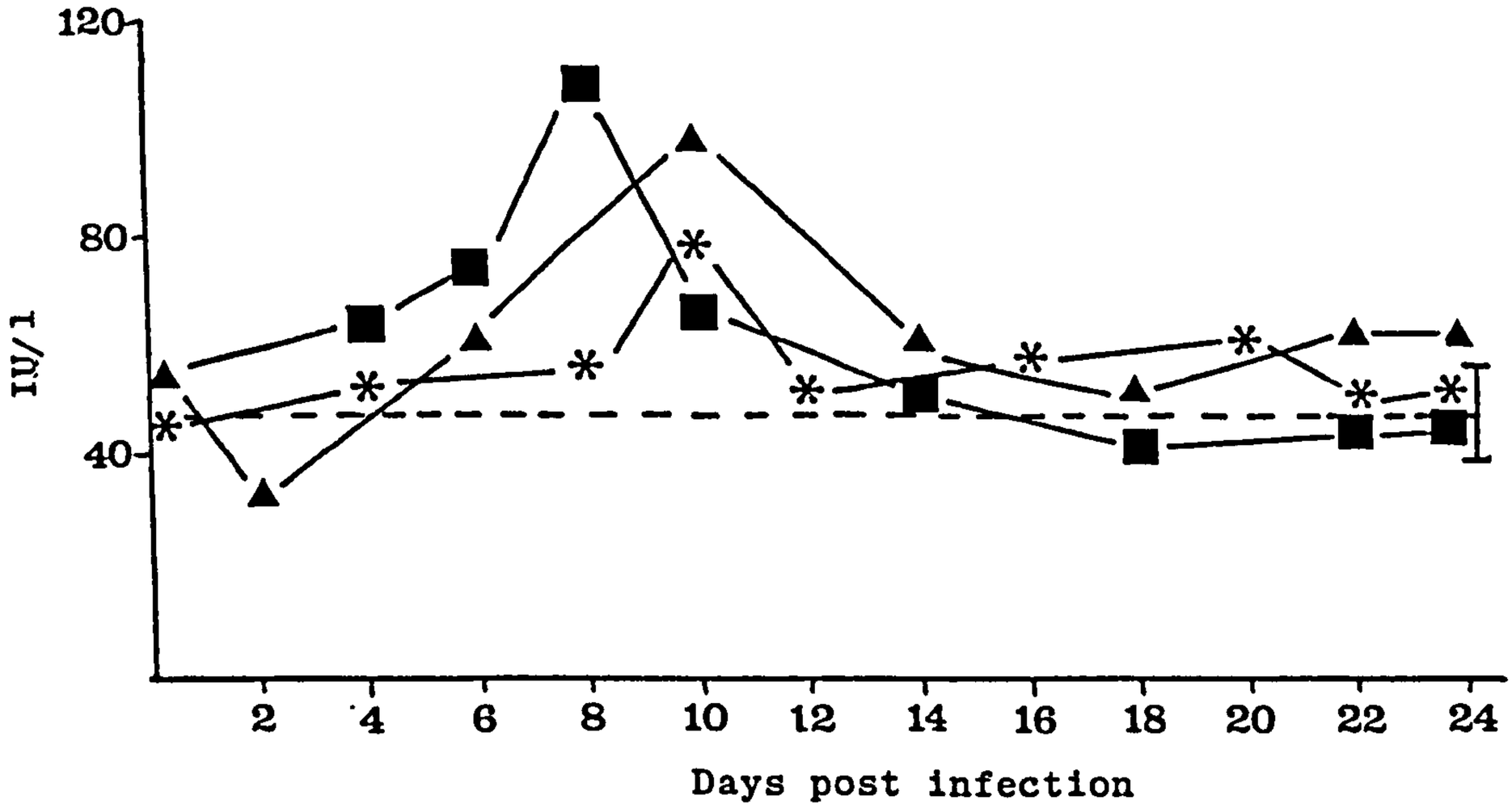


Fig.75 GPT activity in the sera of guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$ of Mopeia viruses.

▲M150 ■M20410 *Z478

------I Normal values

Each point represents the arithmetic mean of 25 guinea pigs.

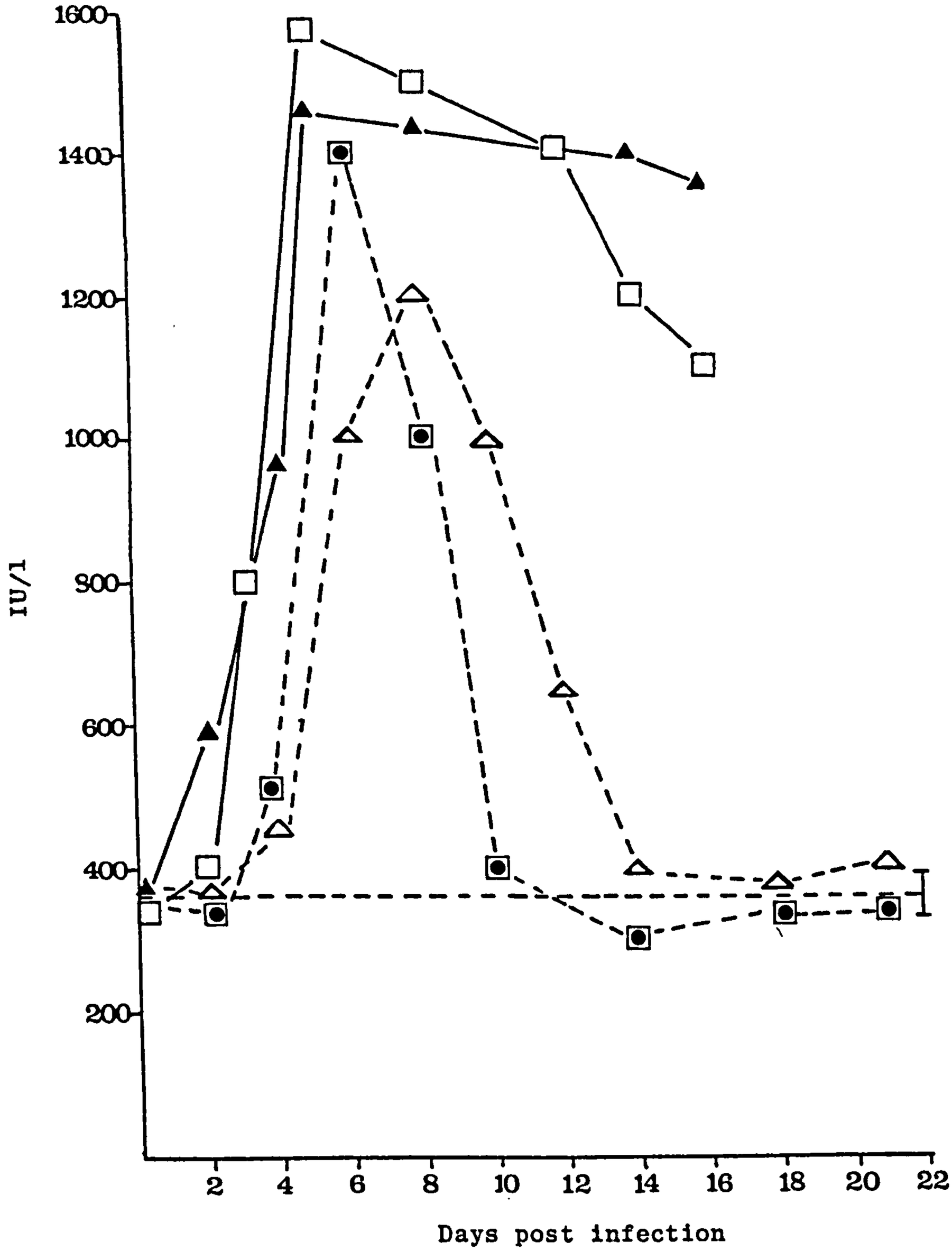


Fig.76 LDH activity in the sera of guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$ of LGA 391 and M152 viruses giving a lethal (——) and non-lethal (---) infection.

□—— □ M152 ▲—— ▲ LGA 391

◉--- ◉ M152 △---- △ LGA 391

-----I Normal values

Each point represents the arithmetic mean of 30 guinea pigs.

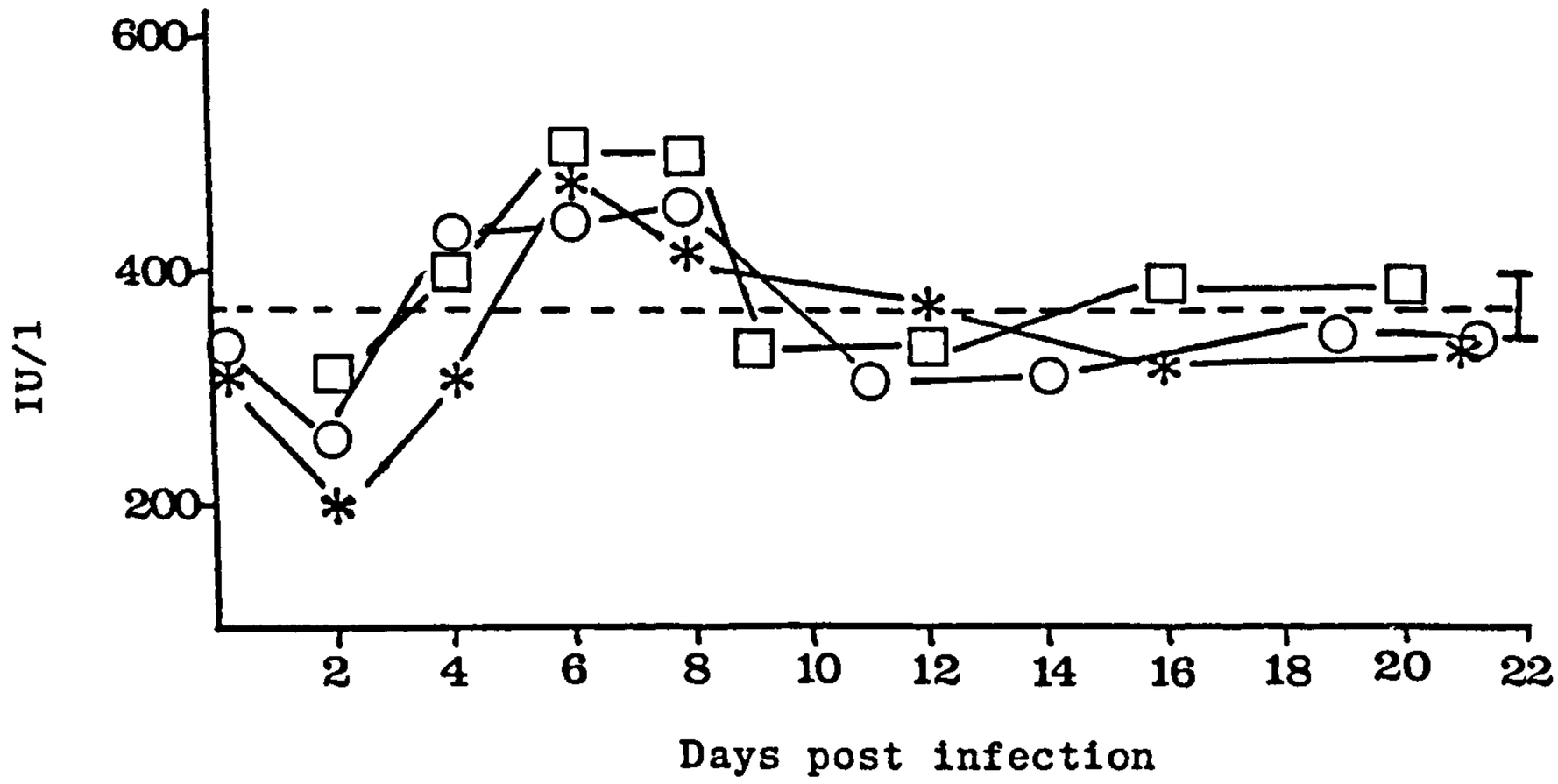
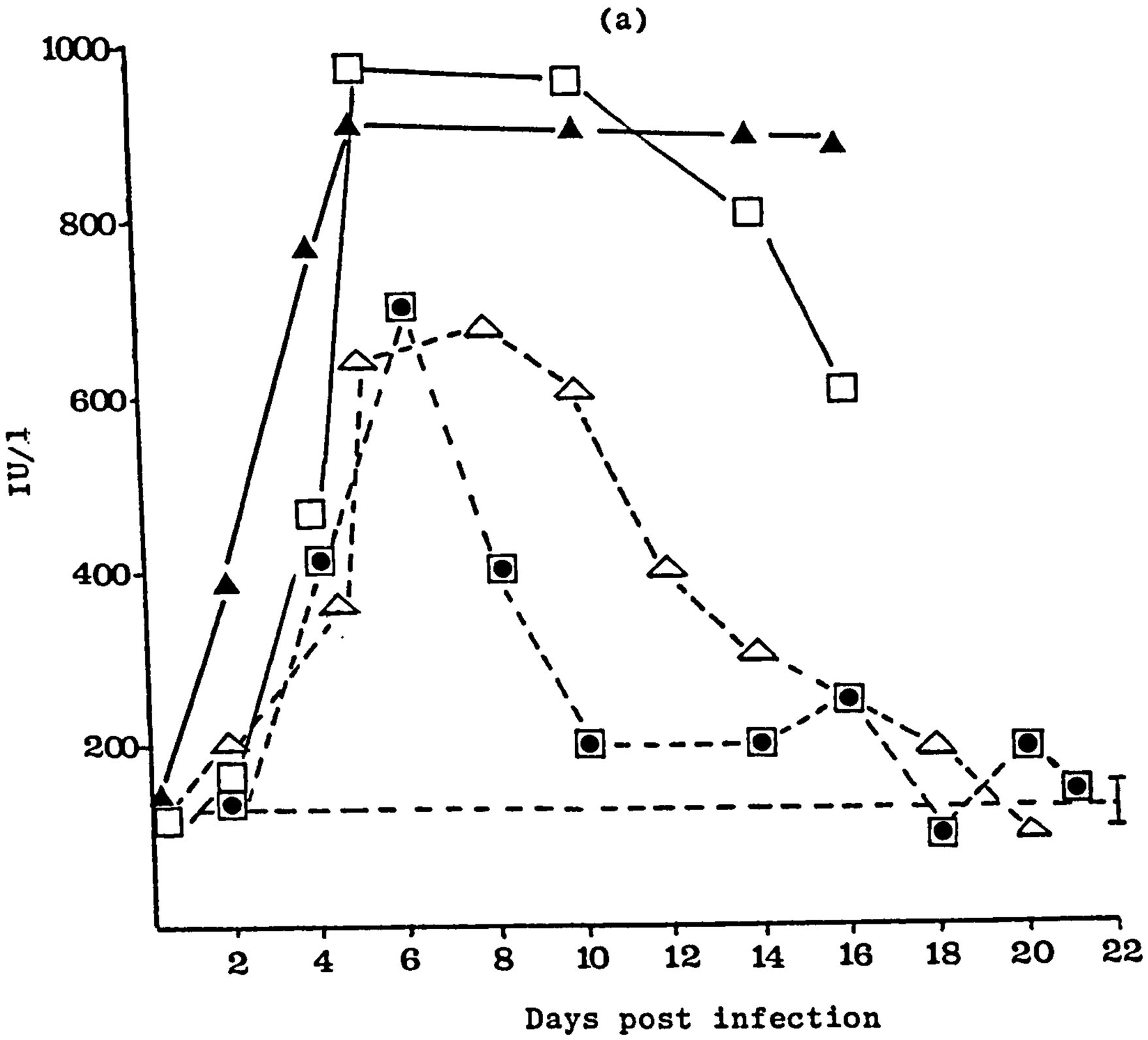


Fig.77 LDH activity in the sera of guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$ of Mopeia virus

✱ M150 ○ M20410 □ Z478

Each point represents the arithmetic mean of 30 guinea pigs.



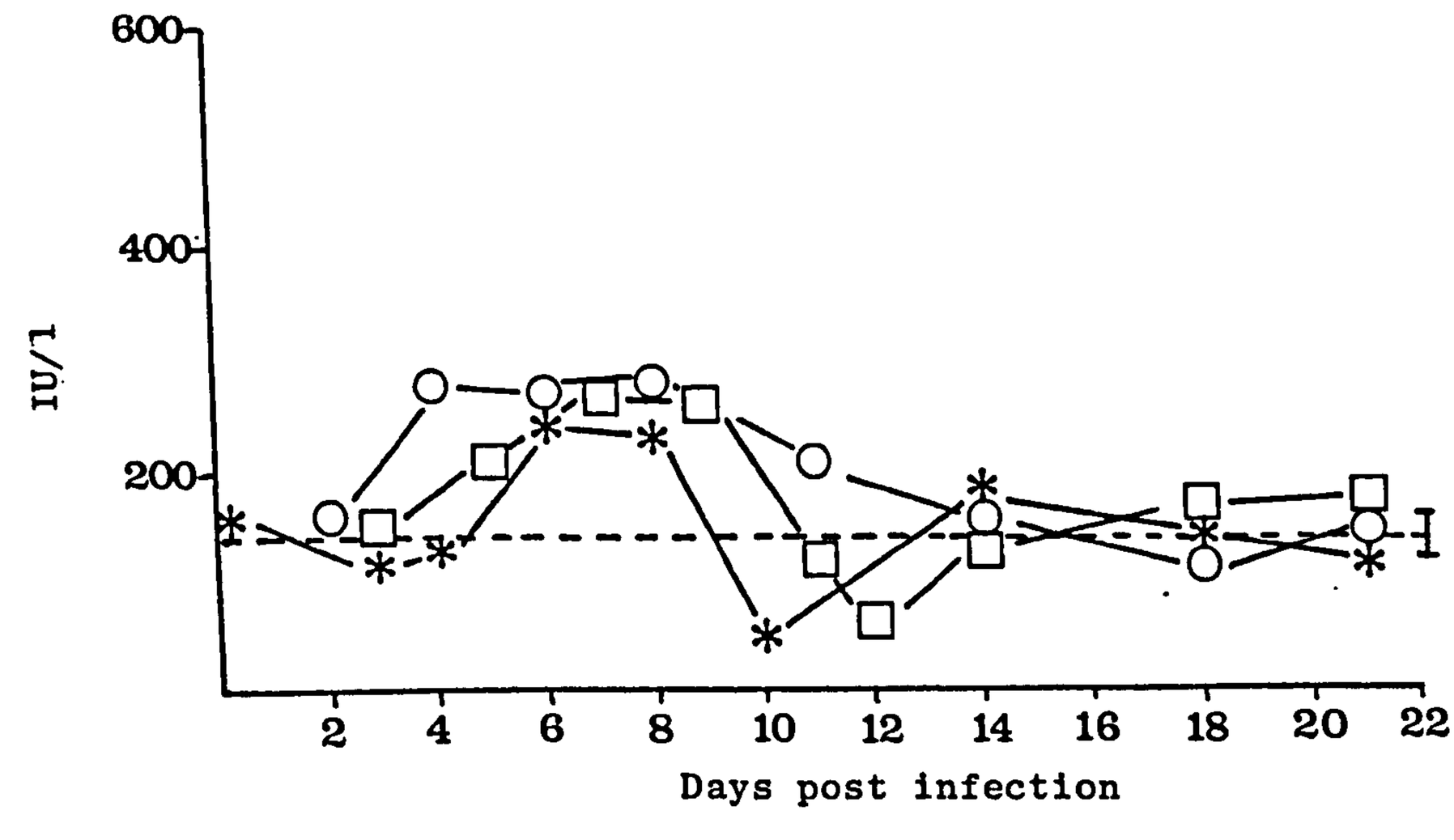


Fig.78 αHBDH activity in the sera of guinea pigs inoculated i.p. with 3 $\log_{10}$ $TCID_{50}$ Lassa and Mopeia viruses.

(a) ▲——▲ LGA 391 lethal; □——□ M152 lethal.

△---△ LGA 391 non-lethal; ◉---◉ M152 non lethal.

(b) * M152 □ Z478 ○ M20410

------I Normal values

Each point represents the arithmetic mean of 30 guinea pigs.

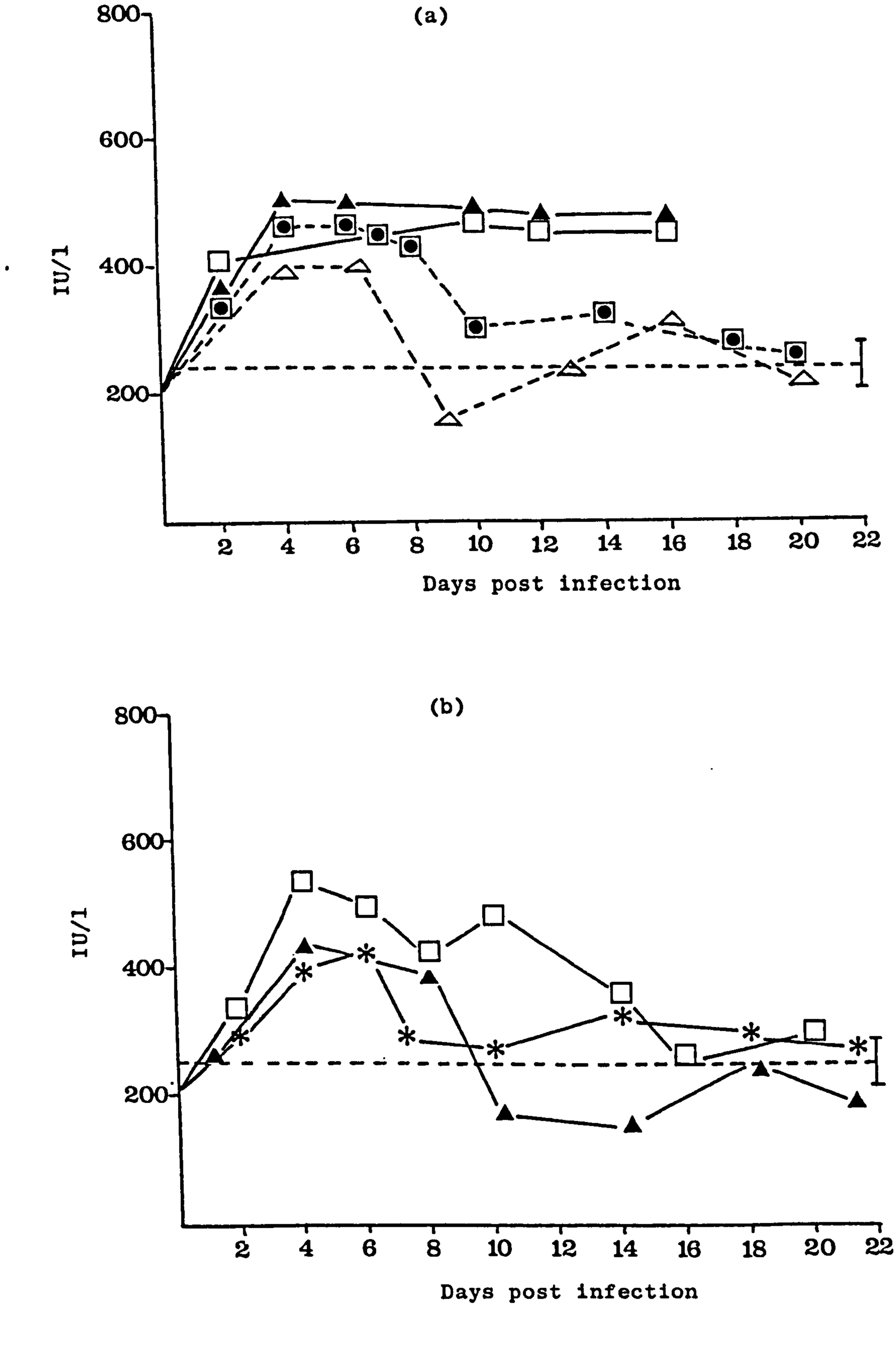


Fig.79 AP activity in sera of guinea pigs infected with Lassa and Mopeia viruses.

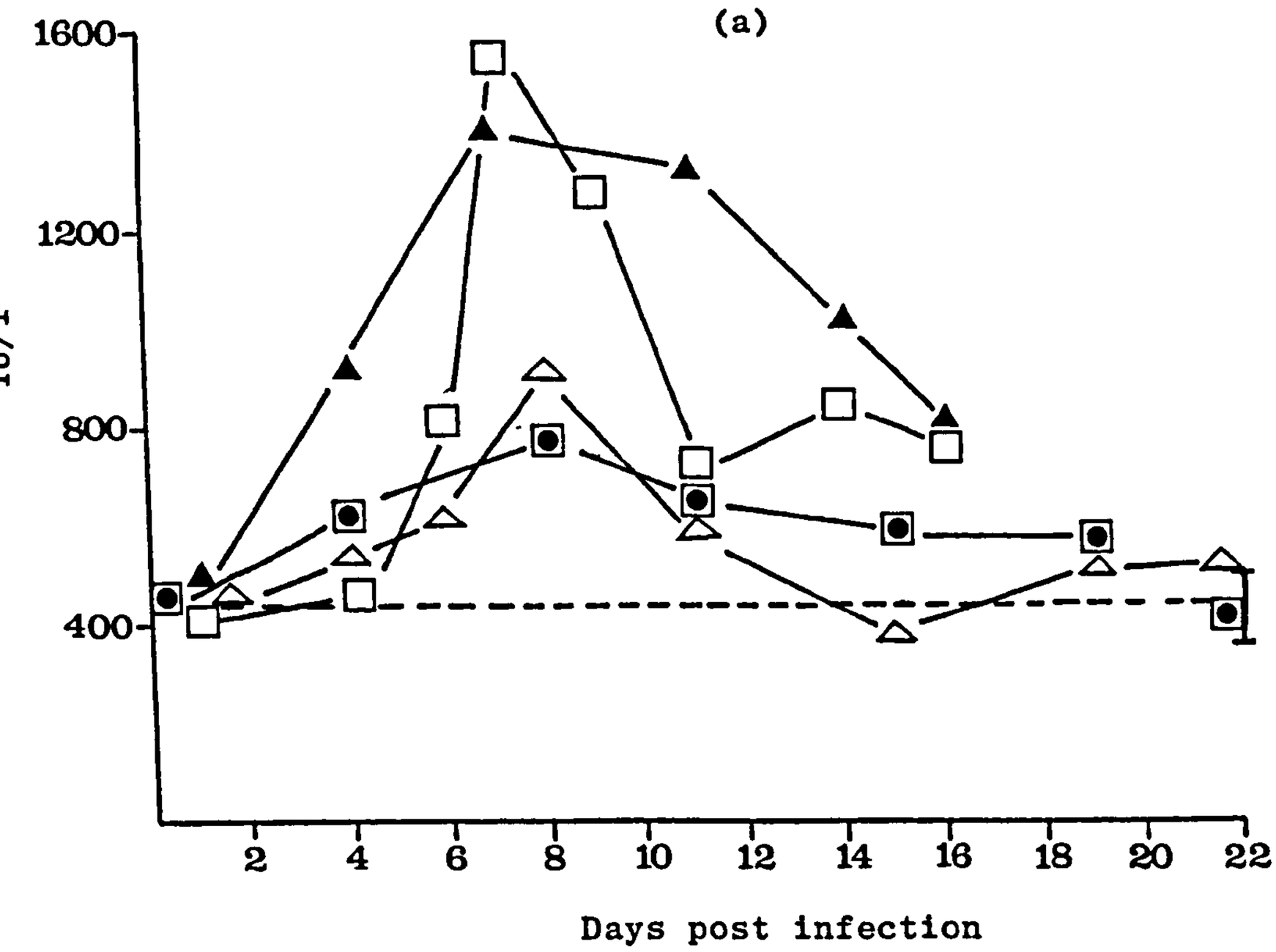
(a) Lethal infections: ▲——▲ LGA 391; □——□M152

Non-lethal infections: △----△LGA 391; ◉----◉M152

(b) □——□M20410; ▲——▲ M150; *——* Z478

------I Normal values

Each point represents the arithmetic mean of 30 guinea pigs.



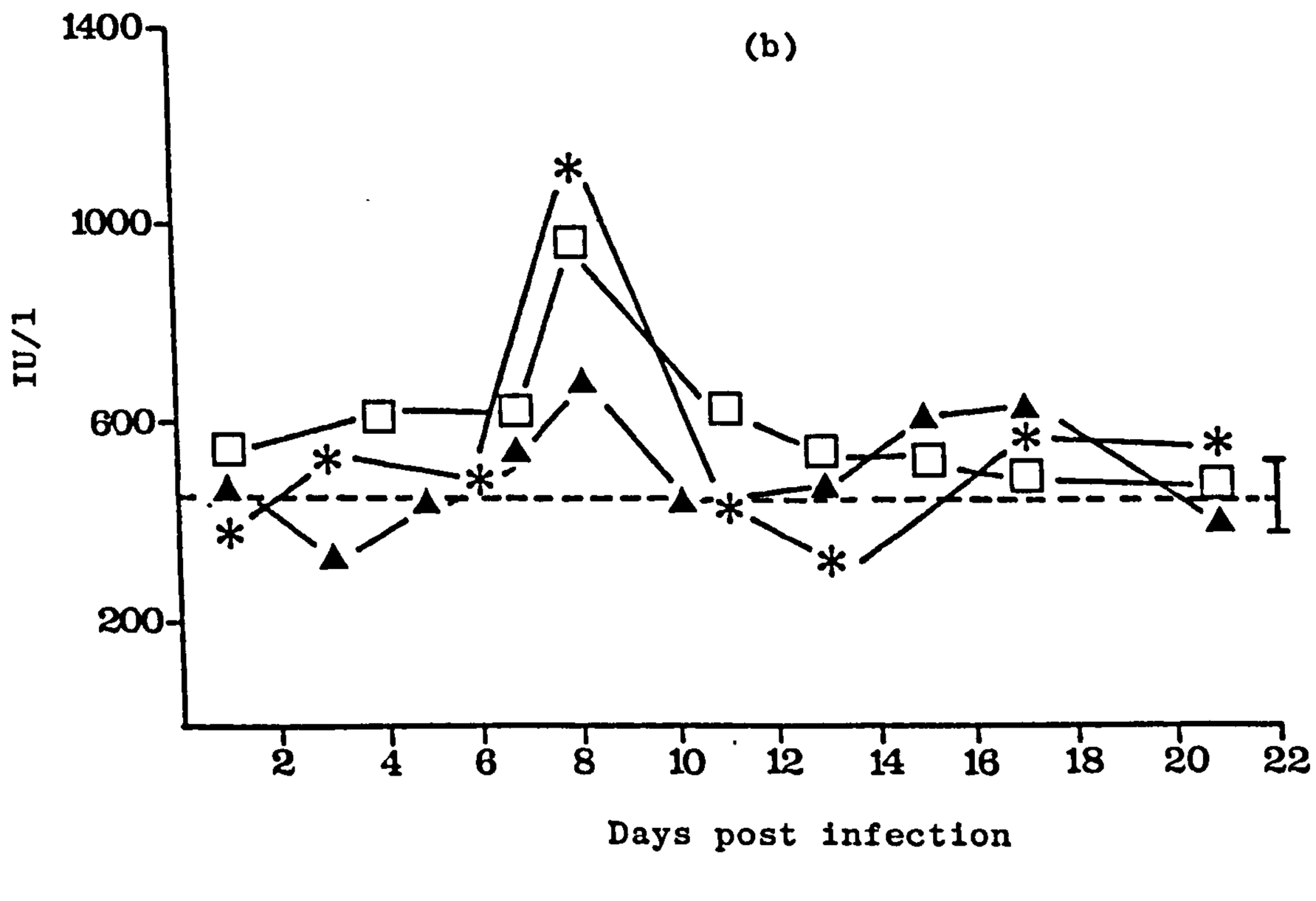


Fig.80 CK activity in sera of guinea pigs infected with Lassa and Mopeia viruses.

(a) Lethal infections ▲—▲LGA 391; □—□M152

Non-lethal △—△LGA 391; ◉—◉M152

(b) □—□M20410 ▲—▲M150 ✱—✱Z478

---------I Normal values (± SE)

Each point represents the arithmetic mean of 30 guinea pigs

TABLE 41. ALTERATION IN ENZYME ACTIVITIES IN SERA OF GUINEA PIGS AFTER INFECTION WITH LGA391 OR M152

Enzyme	Virus	Infection	Peak value	Day	Returned to Normal/Day	Normal value
GOT	LGA391	Lethal	295	5	No	73.4
"	"	Non-lethal	120	8	Yes/14	"
GOT	M152	Lethal	255	5	No	73.4
"	"	Non-lethal	125	8	Yes/18	"
GPT	LGA391	Lethal	108	5	No	46.8
"	"	Non-lethal	60	9	Yes/12	"
GPT	M152	Lethal	114	5	No	46.8
"	"	Non-lethal	90	8	Yes/12	"
LDH	LGA391	Lethal	1450	5	No	361.34
"	"	Non-lethal	1200	8	Yes/10	"
LDH	M152	Lethal	1575	5	No	361.34
"	"	Non-lethal	1400	6	Yes/10	"
αHBDH	LGA391	Lethal	900	5	No	140.3
"	"	Non-lethal	675	8	Yes/14	"
αHBDH	M152	Lethal	975	5	No	140.3
"	"	Non-lethal	700	6	Yes/10	"
AP	LGA391	Lethal	500	4	No	248.8
"	"	Non-lethal	400	4	Yes/10	"
AP	M152	Lethal	475	10	No	248.8
"	"	Non-lethal	450	4	Yes/10	"
CK	LGA 391	Lethal	1300	7	No	448.0
"	"	Non-lethal	650	7	Yes/10	"
CK	M152	Lethal	1500	7	No	448.0
	"	Non-lethal	750	7	Yes/10	"

returning to normal by day 10 p.i. There was no significant alteration with these same viruses in the levels of the remaining serum enzyme and substrates studied.

3.7.6. Histopathology

The distribution and degree of histological damage observed in various tissues was less than would have been predicted on the basis of LGA 391 infectivity and biochemical data.

Lung tissue from ten guinea pigs infected with LGA 391 revealed lesions. There were large coalescing areas of oedema and fibrin formation in alveoli, together with infiltration by macrophages and lymphocytes. Some of the zones were also intensely congested and even haemorrhagic and there was considerable perivascular oedema. There was no necrosis of alveolar walls and no exudate in airway lumina. The alveolar infiltrate contained a higher proportion of lymphocytes at day 15 than on the 8th day.

Lesions were present in the liver and consisted of small areas of sinusoidal haemorrhage, having no consistent lobular distribution. There were no degenerative changes in hepatic parenchymal cells in the haemorrhagic areas.

Three of the ten spleen sections contained necrotizing lesions of varying severity. There were also testicular changes found in two guinea pigs. These consisted of active vasculitis of the blood vessels of the tunica albuginea, tunica vaginalis and the testis substance. Affected vessels had extensive infiltration of the wall and adventitia by lymphocytes and histiocytes though the intima was undamaged.

Four kidney sections displayed acute necrotizing nephritis. Although LGA 391 clearly replicated in the adrenal and salivary glands no remarkable lesions were seen in these tissues.

Brain tissues from the guinea pigs examined contained no lesions.

In the guinea pigs inoculated with M152 liver and lung changes were present and histologically identical to that described for LGA 391.

Groups of tissues examined from guinea pigs inoculated with M150, M20410 and Z478 between days 5 and 40 had no lesions in any organ.

3.7.7. Challenge of Mopeia-immunised guinea pigs with LGA 391

Groups of 10 guinea pigs immunised with either M150, M20410 or Z478 (3 $\log_{10}$ $TCID_{50}$) were challenged with LGA 391 (3 $\log_{10}$ $TCID_{50}$) at various times after immunisation. Studies on their susceptibility to challenge with LGA 391 virus were based on the following parameters:

(a) clinical illness/or mortality

(b) pyrexia

(c) serum viraemia

(d) serum enzymes and substrates

(e) IF antibody

(f) neutralization antibody

(g) histopathology

It was observed that no matter which combination of inoculation routes was used for challenge and/or immunisation, the course of events was identical (Table 42). The latter statement also applies to all three immunising viruses, M150, M20410 and Z478.

TABLE 42. OUTCOME OF IMMUNISATION/CHALLENGE PROTOCOL

	Route of immunisation			
Route of challenge	i.p.	s.c.	i.d.	-ve control
i.p.	protection	protection	protection	No protection
s.c.	"	"	"	"
i.d.	"	"	"	"

The detailed comments on the outcome of challenging an immunised series of guinea pigs with LGA 391 are based on immunising and

challenging s.c. The following data describes the outcome of immunising guinea pigs s.c. with 3 $\log_{10}$ $TCID_{50}$ of M150 and challenging with 3 $\log_{10}$ $TCID_{50}$ LGA 391 at various times post immunisation. These results can equally apply to guinea pigs immunised with M20410 and Z478. Simultaneously, a control group of animals not previously inoculated with Mopeia virus, received the same dose of LGA 391. All groups were kept under observation for 84 days after challenge. Clinical signs and mortality rates were recorded (Table 43, Fig. 81) as were estimations of antibody, virus and serum biochemistry responses.

The susceptibility of guinea pigs immunised with M150 and challenged at different times with LGA 391 are shown in Table 43. Partial protection was observed up to 21 days post immunisation, after which complete resistance was established.

TABLE 43. NUMBER OF CLINICALLY ILL AND/OR DEAD GUINEA PIGS IMMUNISED WITH M150 AND CHALLENGED WITH LGA 391 AT VARIOUS TIMES POST IMMUNISATION

Days post immunisation	No. of animals affected	No. of control animals affected
7	10/10	10/10
14	"	"
21	5/10	"
28	0/10	"
35	"	"
42	"	"
56	"	"
70	"	"
84	"	"

To analyse the origin of protection, guinea pigs at day 48 post M150 immunisation were challenged s.c. with LGA 391 (3 $\log_{10}$ $TCID_{50}$), since no cross-reacting neutralization antibodies were detectable in guinea pigs

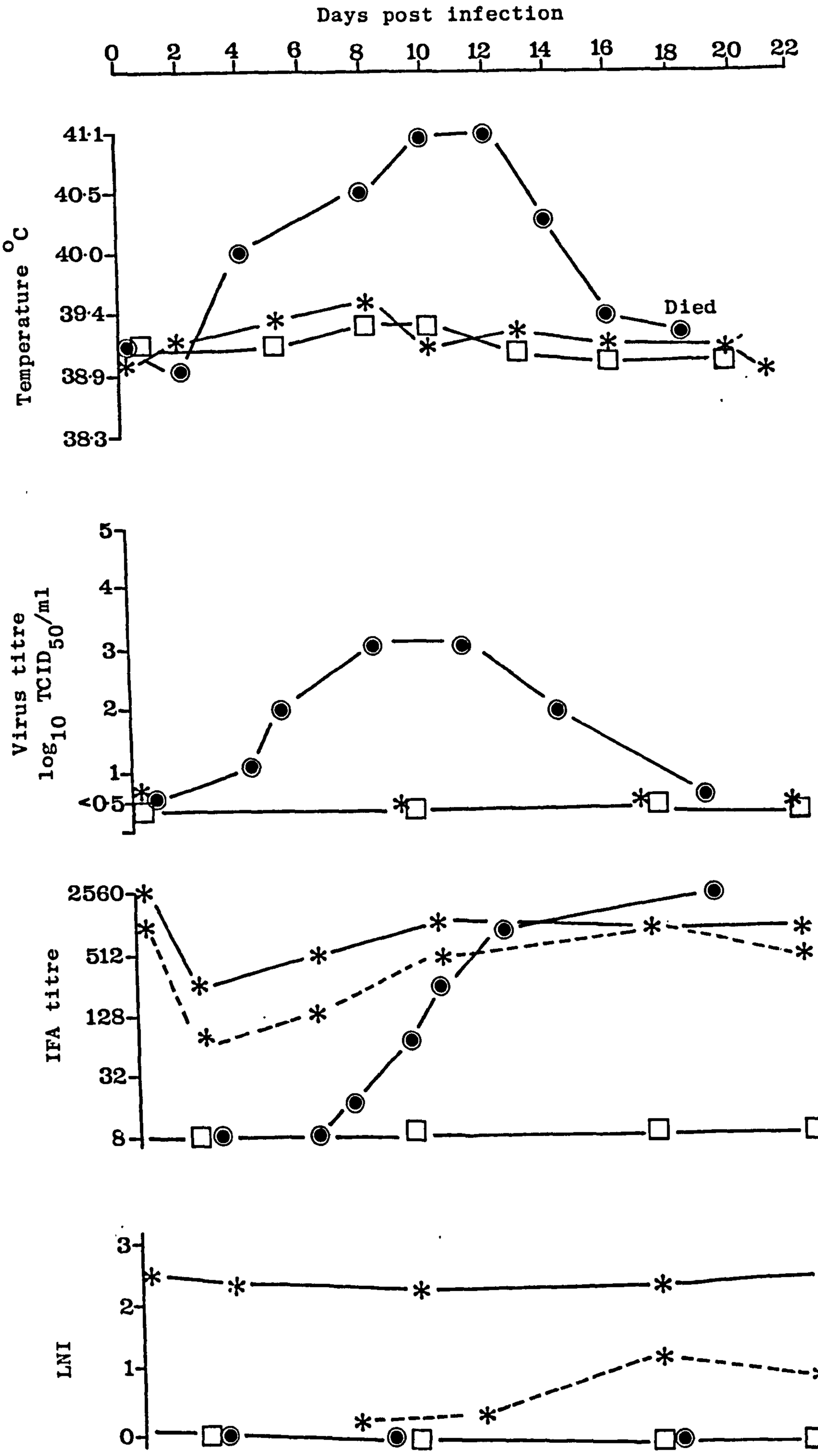


Fig.81 Course of LGA 391 challenge of guinea pigs 48 days after immunisation with M150

●LGA 391 only infected guinea pigs (+ control)

□Uninfected guinea pigs (-ve control)

*LGA 391/M150 test guinea pigs
For the development of IF and N antibody

*——*anti-Mopeia *---*anti-LGA 391

inoculated only with M150 (see Section 3.7.4 and Table 39). The level of virus found in the blood and tissue of LGA 391 control guinea pigs was high (Figs. 69 and 71). In contrast, no Lassa virus could be isolated from animals immunised with M150 virus. With guinea pigs infected with M150 alone no virus could be detected. Although viral assays were performed in Vero cells, various tissue homogenates from challenged animals were also put into adult mice i.c. and guinea pigs i.p., with negative results.

The results (Fig. 81) also demonstrated the immunological responses of M150 immunised guinea pigs when challenged 48 days later with LGA 391. The IFA levels to Mopeia and LGA 391 before challenge were similar (1/1028 - 1/2056). Homologous neutralization antibodies to M150 were estimated to give an LNI of 2.4 while heterologous antibodies to LGA 391 were not demonstrated. Normal guinea pigs infected with M150 did not have any demonstrable neutralization antibodies to LGA 391 virus. M150 immunised animals challenged at 48 days post infection with LGA 391 gave rise to demonstrable anti-neutralizing Lassa antibodies 6-10days post challenge, reaching an LNI of 1.4 after 21 days.

As a result of challenge no febrile illness, no serum viraemia was recorded throughout the post challenge period of study. Those serum enzymes expected to alter (see Section 3.7.5.), GOT, GPT, LDH, αHBDH, AP, and CK did not show any alterations from normal.

The gross pathology and histopathology carried out on liver, spleen, heart, lymph nodes, kidney, adrenals, brain and muscle revealed no lesions or other abnormalities. Urine removed at autopsy from LGA 391/M150 animals contained no Lassa or Mopeia viruses.

3.8. PRELIMINARY STUDIES ON TRANSMISSION OF LASSA AND MOPEIA VIRUSES TO RHESUS MONKEYS

Although Rhesus (Rh) monkeys have not been implicated in the transmission of Lassa infections, it was thought important to infect

monkeys experimentally with Lassa and Mopeia viruses to define their comparative pathogeneses and determine whether this would provide a useful model for evaluating methods of therapy and protection for use in human Lassa virus infection.

3.8.1. Transmission of LGA 391 virus

A male Rhesus monkey at 6.8 Kg was inoculated i.p. with 0.1 ml of LGA 391 tissue culture fluid containing 3 $\log_{10}$ $TCID_{50}$. The progress of the disease was monitored daily by rectal temperature and a blood sample taken.

3.8.1.1. Clinical observations

Following a 2-day incubation period the monkey became febrile on about the third day post infection with a temperature ranging from 40.2 - 40.5°C (Fig. 82). The pyrexia persisted until the terminal stages of the infection when the temperature became sub-normal (38.6°C) on days 9-10. By day 4 the monkey was quiet, it was not eating or drinking and normally lay on the floor of the cage only sharply responding in the early stages to provocation (days 4-6 p.i.). By day 6 no amount of provocation stimulated any response. No exterior markings or rash became evident throughout the duration of the illness. Excessive salivation was evident during days 5-7 but contained no virus. Body weight was reduced by 10-15 per cent. Slight diarrhoea was evident on days 6 and 7 post infection and death occurred on day 9.

3.8.1.2. Virus levels in the blood

Virus was detected in the blood on day 4 reaching maximum levels of $10^{4.6}$ $TCID_{50}$/ml on day 6 which was maintained until death.

3.8.1.3. Virus levels in the tissues

The concentration of infectious virus recovered from tissues showed high concentrations of Lassa in most organs of the body (Table 43a). It was not possible to determine whether some of the organs contained Lassa

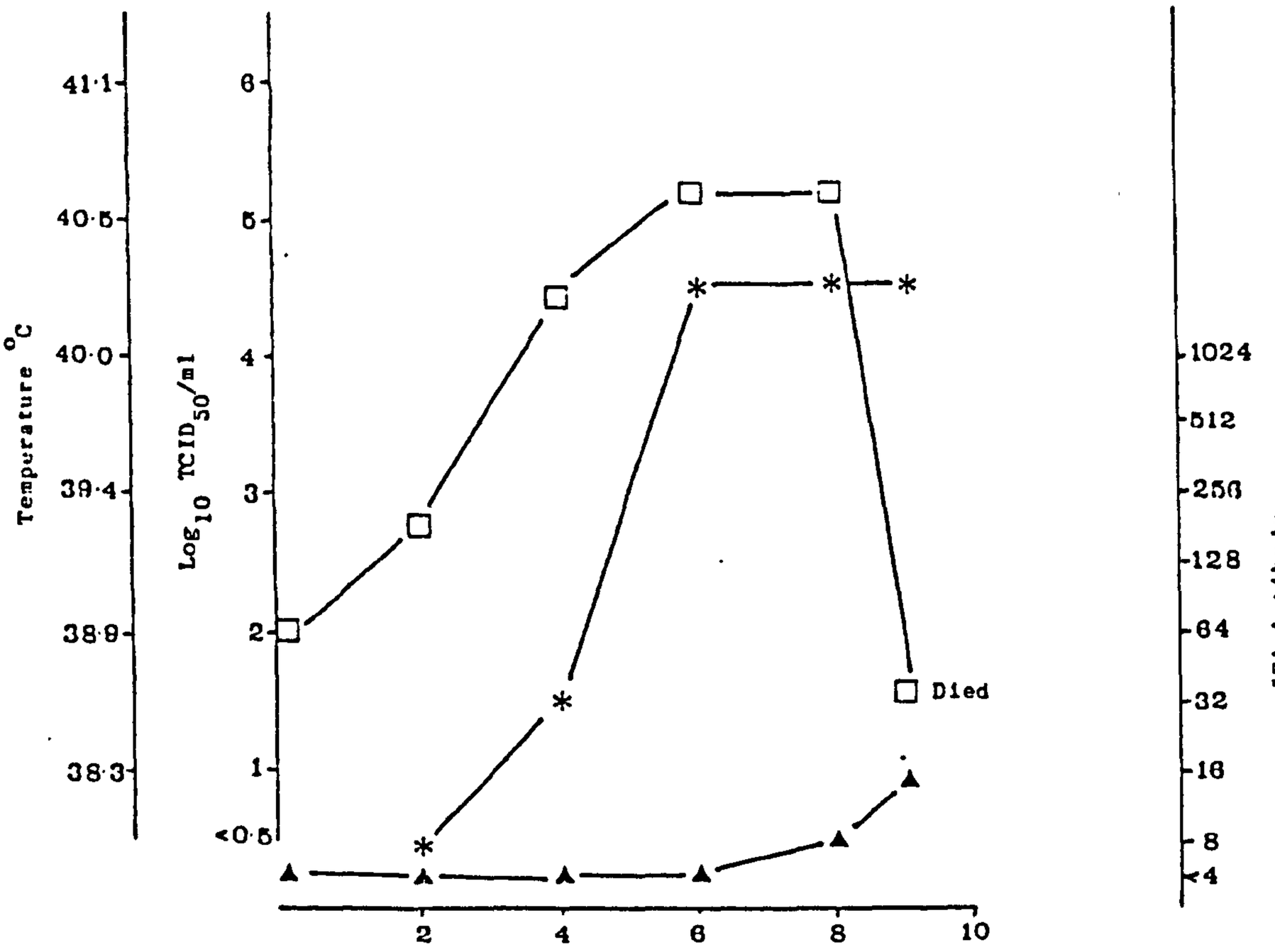


Fig. 82. Temperature, viraemia and immunofluorescent antibody responses in Rhesus monkeys infected i.p. with 3 $\log_{10}$TCID$_{50}$ of Lassa LGA 391.

▲ IF antibody (IgG) □ Temperature * Virus

virus because of the high concentrations of virus present in the blood. However, virus was recovered in titres higher than those in blood from all visceral tissue tested including lung, adrenal glands, spleen, pancreas and lymph nodes. Although the faeces never contained any virus, some was isolated in the urine ($10^{4.5}TCID_{50}/ml$) and peritoneal fluid ($10^{2.3}TCID_{50}/ml$).

TABLE 43a. LEVELS OF VIRUS IN VARIOUS TISSUES FROM RHESUS MONKEY EXPERIMENTALLY INFECTED WITH LASSA VIRUS LGA 391

Organ	Viral titre at time of death (day 10)
Blood	4.6
Adrenal glands	8.2
Spleen	7.8
Liver	8.4
Thymus	6.4
Lungs	8.4
Heart	8.3
Pancreas	5.6
Salivary glands	7.7
Bladder	4.5
Duodenum	8.4
Pleural fluid	2.3
Kidney	8.7

N.B. Data on $\log_{10} TCID_{50}/ml$ or $\log_{10} TCID_{50}/g$

3.8.1.4. Gross necropsy findings

There were no significant gross pathological lesions or any obvious haemorrhagic manifestations. The lungs did show some degree of consolidation. Peritonitis was in evidence together with early adhesions which involved the liver and spleen. The liver and spleen were slightly enlarged and friable. Mesenteric lymph nodes were also enlarged.

3.8.1.5. Histopathology

In the liver there was evidence of hepatocellular necrosis accompanied by slight infiltration of inflammatory cells. The small foci of necrosis was randomly distributed throughout the parenchyma. In a typical focus hepatocytes demonstrated varying degrees of degeneration with some evidence of eosinophilic spherical inclusion bodies. Mitotic figures were seen in some hepatocytes together with binucleation and nuclear pleomorphism.

The kidney showed renal tubular necrosis. Tubular lumina contained necrotic cells with aggregates of degenerating nuclei. There was limited congestion, small inter-tubular haemorrhages in the blood vessels of the cortex and outer medulla. Inclusion bodies could not be demonstrated. Cellular infiltration was absent.

The splenic white pulp contained lymphoblastic re-population of the B-cell region but still contained eosinophilic necrotic material. Lymphoid tissue necrosis of the Malpighian corpuscles was present to some degree and lymphocytic depletion appeared evident in some follicles together with some congestion of the red pulp.

There was generalised depletion of lymph nodes. The mesenteric lymph node contained intracellular inflammation, possibly macrophages or reticuloendothelial cells. There was some germinal centre necrosis.

The lungs demonstrated moderate foci of necrosis of septa with some intra-alveolar oedema formation and haemorrhage into the alveoli. There was evidence of interstitial pneumonia. It was characterized by alveolar wall thickening, the result of deposition within septa of eosinophilic material and mononuclear cell infiltrations. The septa also contained cellular debris, which was suggestive of endothelial cell necrosis, mixed

with infiltrate. The epithelium of bronchi and bronchioli remained unaffected. There was thrombosis and fibrin deposition in pulmonary vesicles. There was no damage found to the walls of affected.

The adrenal glands contained multifocal adenocortical necrosis principally in the zona reticulinus and zona fasciculata. Within and adjacent to foci of necrosis were adenocortical cells with eosinophilic cytoplasmic inclusions. There was little congestion of the blood vessels of the cortex and medulla.

The small intestine had erythrocytes and macrophages in the lamina propria. Minimal perivascular mononuclear cell infiltration was observed in the sub-mucosa.

Within the gastrointestinal tract there was no congestion observed in the ileum or large intestine. There was no necrosis of the surface epithelium, crypt tissue or lamina propria. There was a little necrosis found in the mucosa which involved in some instances the Pey er's patches. No irregularity was found in the villi.

The myocardium showed acute myocarditis with vacuolar degeneration and necrosis of myocardial fibres. There was moderate inflammatory infiltration primarily composed of polymorphonuclear leukocytes and mononuclear cells.

No changes were found in other tissues, namely the pancreas, salivary glands or skeletal muscle.

3.8.1.6. Clinical Biochemistry

Normal values of plasma enzymes and substrates were first calculated in a series of samples obtained from normal Rhesus monkeys (Table 44).

TABLE 44. NORMAL VALUES OF SERUM ENZYMES AND SUBSTRATES IN RHESUS MONKEYS

Enzyme/Substrates	Normal values*	±SE	Units
GOT	50.2	12.50	IU/l
GPT	28.2	2.50	"
LDH	850.0	28.70	"
α-HBDH	585	54.74	"
CK	287.65	41.02	"
γ-GT	36.70	5.30	"
AP	458.37	26.25	"
Creatine	0.68	0.12	mg/100ml
Urea	38.39	4.82	"
Total protein	8.4	1.2	"
Triglycerides	63	5.3	"
Cholesterol	95.5	5.2	"

*Each value represents analysis of 30 samples.

Both GOT and GPT were elevated during LGA 391 infection. Elevation of GOT began to rise on day 1 p.i. reaching a peak level of 348 IU/l on day 8 and 9. GPT levels rose on day 4 p.i., reaching 212 IU/l on day 8 (Fig.33a). Normal value in Rh monkeys of GOT and GPT are shown in Table 44).

The isoenzymes LDH and αHBDH both decreased from their normal levels. In the case of LDH the serum value decreased from 1378 to 547 IU/l by day 4 p.i. and by day 9 the level had risen to 3528 IU/l (Fig. 84a). A similar pattern was observed with αHBDH which remained stable at 836 IU/l for the first 2 days, falling to 368 IU/l by day 4. The level of αHBDH rose to 2980 IU/l by day 9 p.i. In Rh monkeys the normal values for LDH are 850 IU/l +/- SE 28.7, and for αHBDH 585 +/- SE 300.

Creatine kinase (CK) activity had a biphasic response. During the first phase its value rose from a normal value of 230 +/- SE 41 to 778 IU/l by day 2 p.i. Between day 2 and day 4 it returned to normal. The CK value reached its peak of 1400 IU/l by day 9 (Fig. 85a).

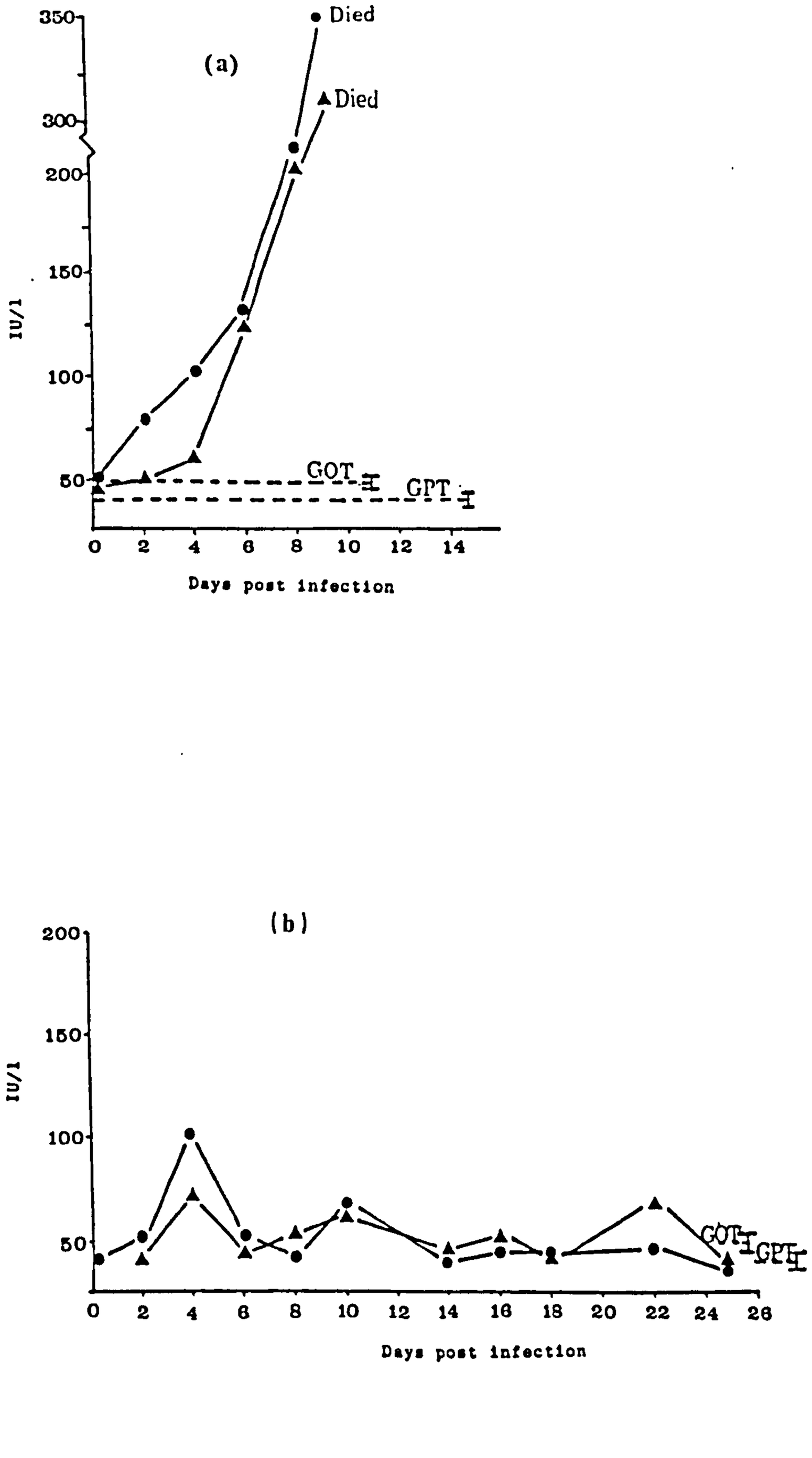


Fig. 83 Glutamine oxaloacetate transaminase●(GOT) and Glutamine pyruvate transaminase▲(GPT) activity in the serum of Rhesus Monkey after infection with (a) Lassa LGA391 (b) Mopeia M150

--------I normal range

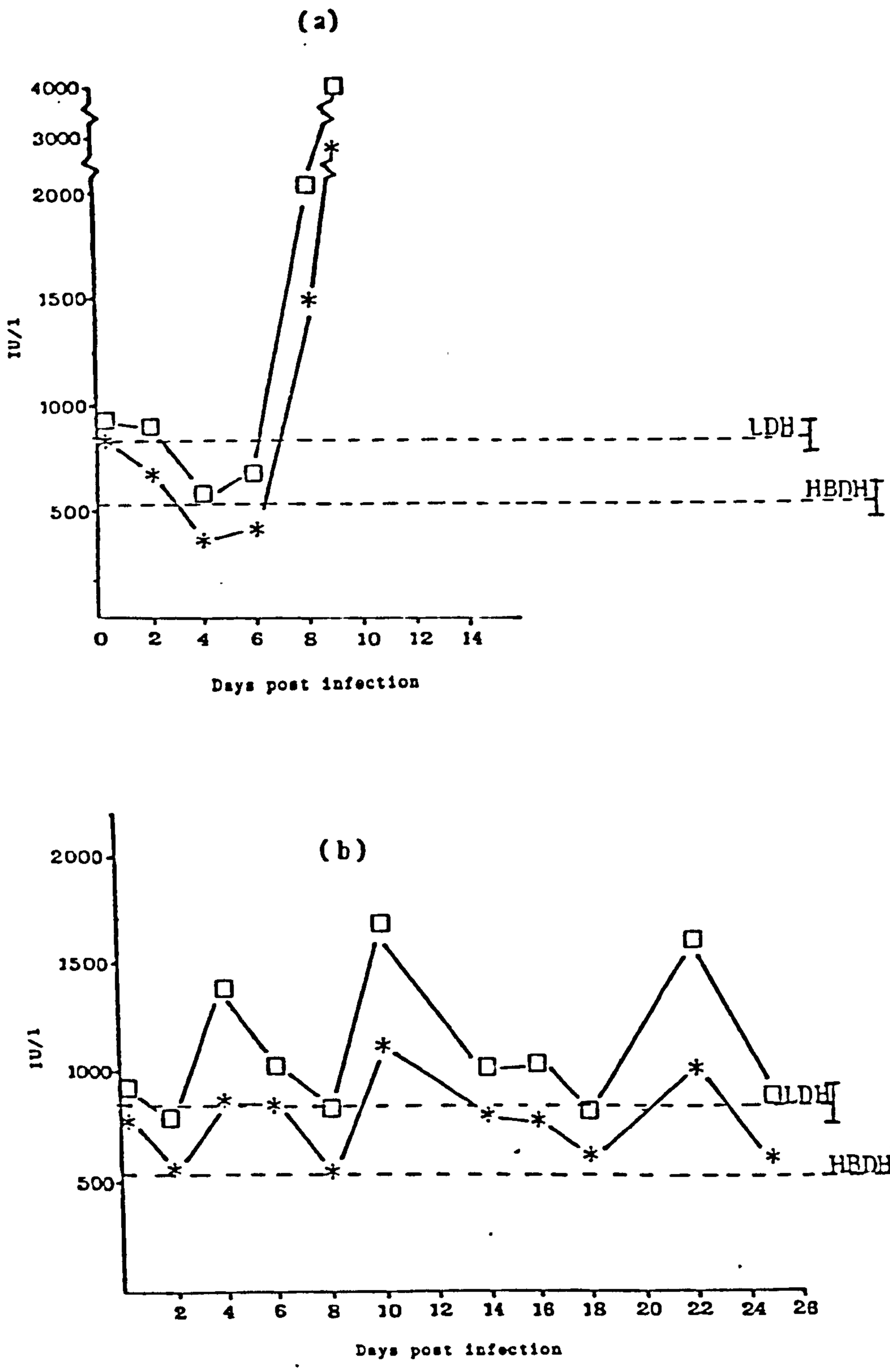


Fig. 84 Lactate dehydrogenase □(LDH) and α-hydroxybutyrate dehydrogenase *(α-HBDH) activity in the serum of Rhesus monkey after infection with (a) Lassa LGA391 and (b) Mopeia M150

--------I Normal range

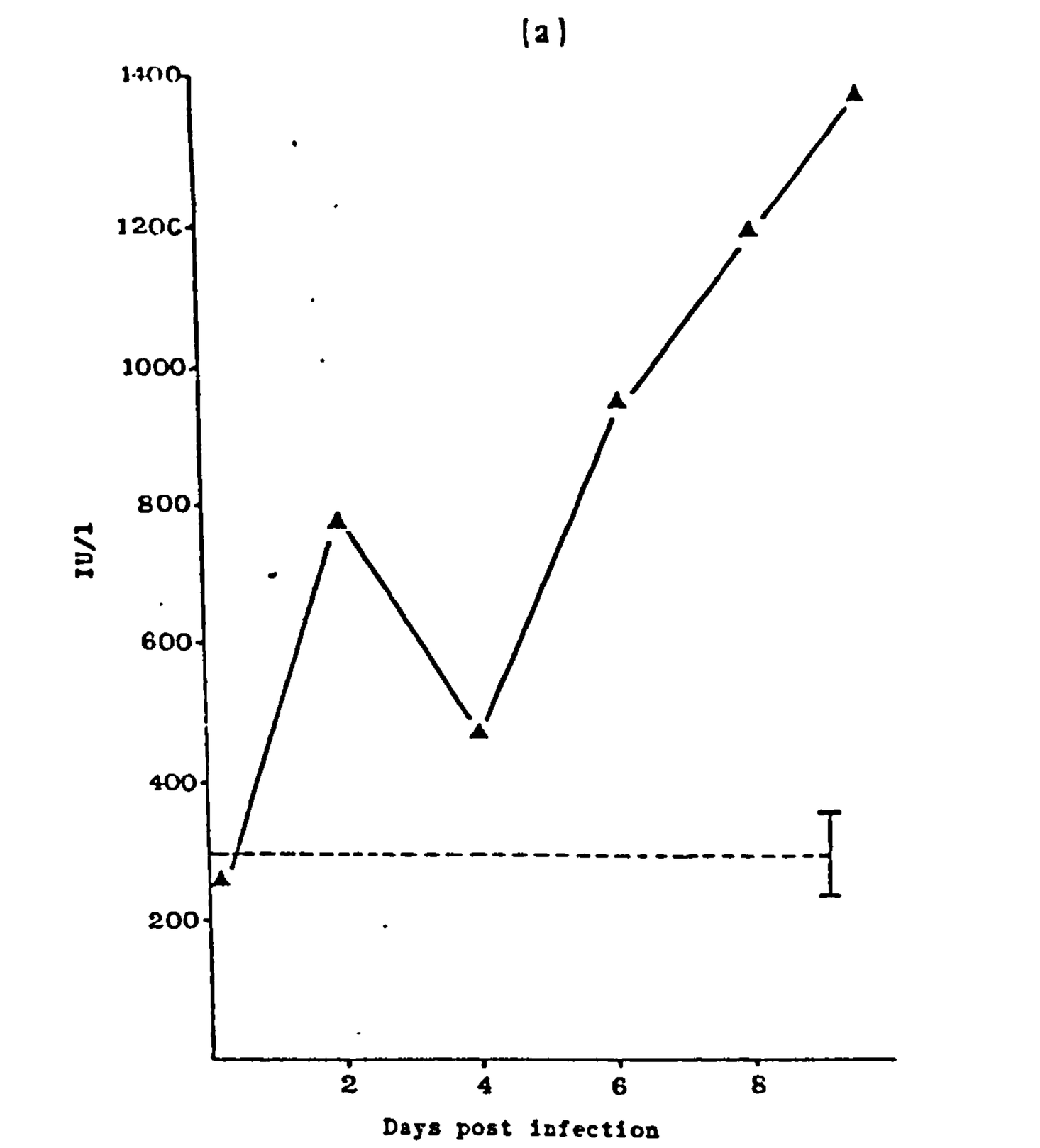


Fig. 85 Creatine kinase (CK) activity in the serum of Rhesus Monkeys after infection with (a) Lassa LGA391 and (b) Mopeia 150

---------I normal range

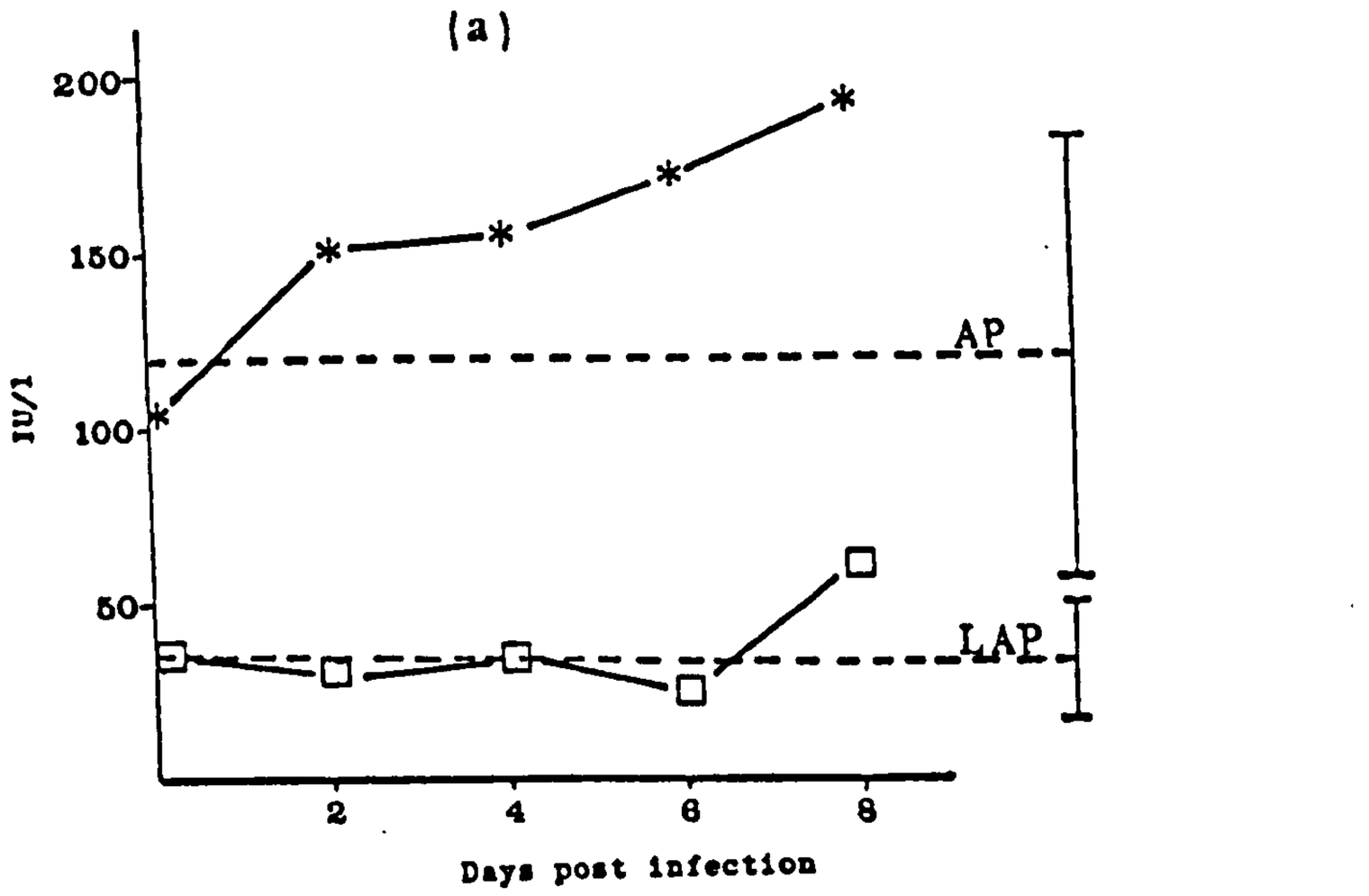


Fig. 86 Alkaline phosphatase (AP) and Leucine amino peptidase (LAP) activity in the serum of Rhesus monkeys after infection with (a) Lassa LGA 391, and (b) Mopeia M150

--------I Normal values * AP □ LAP

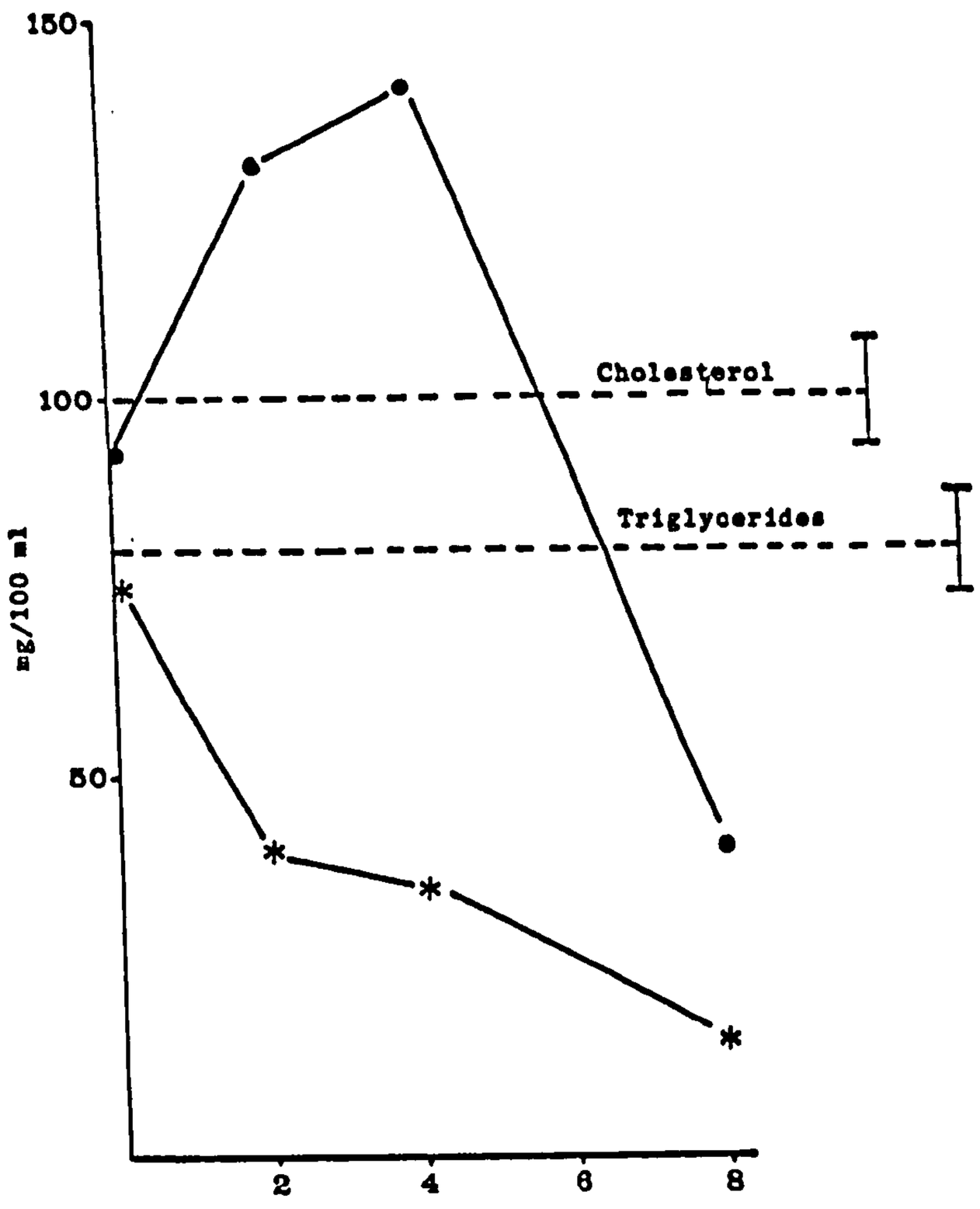


Fig.87 Cholesterol and Triglyceride activity in the serum of Rhesus monkeys after infection with Lassa GA 391

● Cholesterol * Triglycerides

-------I Normal values

Alkaline phosphatase (AP), Glutamyl transpeptidase (GT) and Leucine amino peptidase (LAP) (Fig. 86) activities all rose above their normal values as the course of infection progressed.

Of the substrates investigated there was an initial rise in Cholesterol level from 99 mg/100 ml to 132 mg/100 ml in the first 4 days of infection followed by a decrease to 47 mg/100 ml by day 8-9 (Fig. 87). Normal values for Rhesus monkeys are 95.5 SE+/- 5.2 mg/100 ml.

Triglyceride serum levels decreased throughout the infection from 65 mg/100 ml to 15.7 mg/100 day 8 p.i. (Fig. 87). Normal values range from 63 +/- SE 5.3 mg/100 ml.

Total serum protein levels increased marginally from 8.4 to 15.2 mg/100 ml at death of the monkey .

Serum urea and creatine levels did not significantly alter from normal values throughout the infection.

3.8.1.7. Antibody levels in the blood

No CFT or neutralizing antibodies were detected within the 9 day period of infection. An immunofluorescent IgG reciprocal antibody titre of 1/8 was noted on day 8 and 1/16 on day 9. No immunofluorescent IgM antibody was found.

3.8.2. Transmission of Mopeia 150 virus

A male Rhesus monkey weighing 6.6 Kg was inoculated i.p. with 0.1 ml stock tissue culture fluid containing 3 $\log_{10}TCID_{50}$ M150 virus. The course of the infection was monitored in detail over the first 26 days p.i. and the results recorded.

3.8.2.1. Clinical observations

A mild febrile illness became apparent during the first 8 days of infection. Pyrexia reached a peak of 40.4°C on day 8 but returned to 39.7°C by day 10 and 34.3°C by day 16. Thereafter it fluctuated between 39.3 and 39.9°C till day 26 (Fig. 88). At no time was any distress noted,

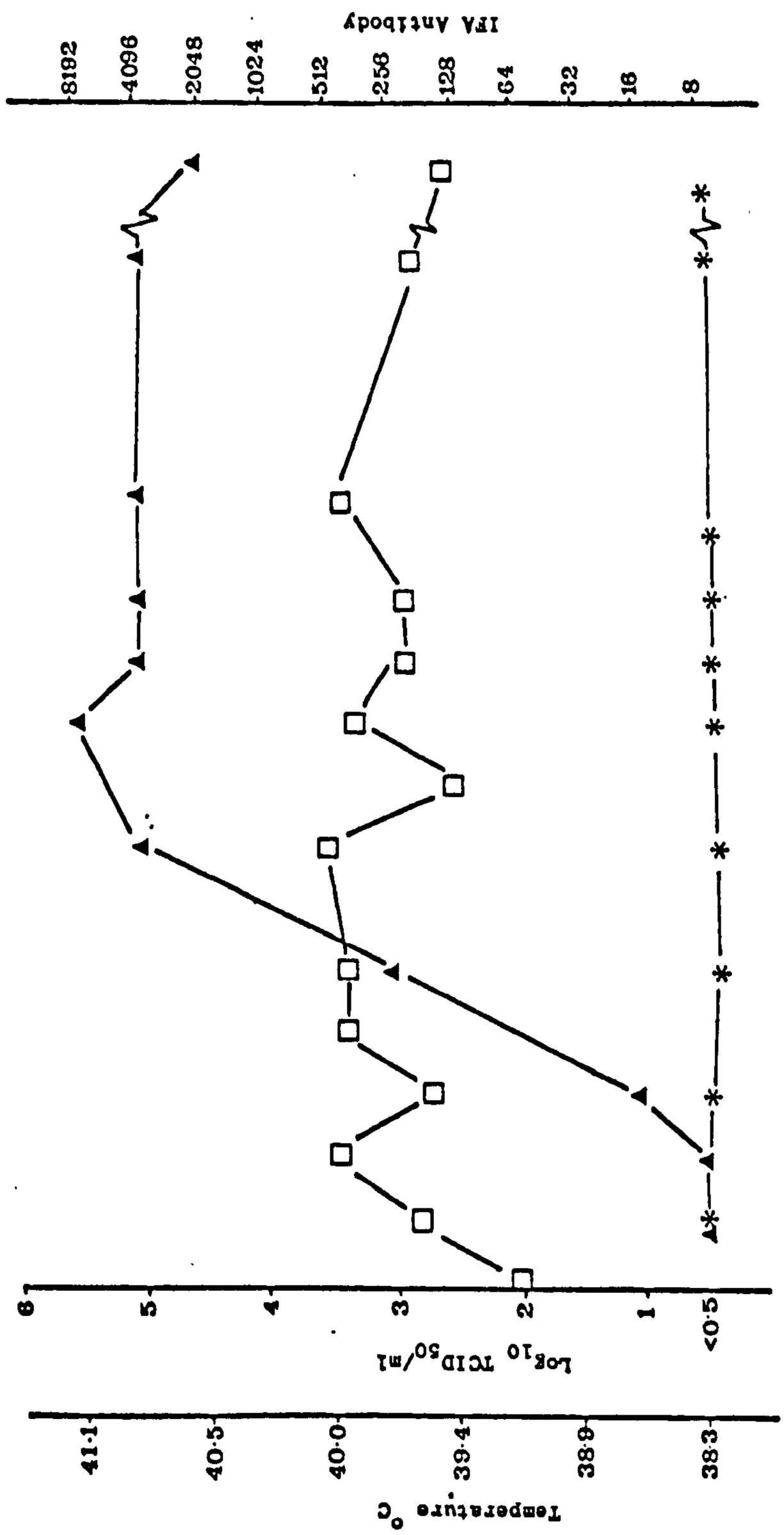


Fig. 88 Temperature, viraemia and immunofluorescent antibody responses in Rhesus monkeys infected i.p. with 3 $\log_{10}$ $TCID_{50}$ of Mopeia virus (M150)

▲ IF antibody (IgG) □ Temperature * Virus

water and food intake remained consistent throughout. Responses to provocation never faltered throughout the study period. No salivation, or diarrhoea was evident. Body weight continued to increase normally.

3.8.2.2. Virus levels in the blood

Virus was not detected throughout the 26-day study period.

3.8.2.3. Clinical biochemistry

The serum GOT and GPT were only slightly altered during Mopeia infection. Both transaminase levels began to rise on day 2 p.i. The GOT reached 110 IU/l on day 4 and GPT increased to 78 IU/l also on day 4 (Fig. 83b).

The isoenzymes LDH and HBDH fluctuated about their normal values throughout the 26 day study period (Fig. 84b).

Creatine kinase did show a high degree of activity between days 4 and 16 p.i. (Fig. 85b). The CK level also increased from 240 IU/l on day 0 to 1121 IU/l on day 4, decreasing to 580 IU/l on day 6 and returning to 1283 IU/l on day 8 where it remained to day 14 p.i. Normal values were observed from day 16 p.i. onwards.

The other enzymes AP, GT and LAH activity remained within normal values throughout the course of infection .

None of the substrates studied significantly altered from normal values.

3.8.2.4. Antibody levels in the blood

The serologic responses, as measured by IFA anti-IgG were first detectable on day 6 (1 : 16) and reached their peak on day 14 p.i. at 1 : 4096.

Complement fixation antibodies against Mopeia and LGA 391 strains were not detected by day 26, but further studies demonstrated a reciprocal CF antibody of 1 : 16 at day 32 which remained unaltered at day 56 p.i.

Neutralizing antibodies against M150 appeared on day 46 p.i. at an LNI of 1.0. Before this date no neutralizing antibodies could be detected

against either Mopeia or Lassa virus strains. No anti-Lassa neutralization antibodies could be detected at day 46.

3.8.2.5. Challenge with M150

The Rhesus monkey first inoculated with M150 was challenged intraperitoneally a second time with 0.1 ml of stock M150 virus (3 $\log_{10}TCID_{50}$) on day 56. At the time of challenge the clinical, immunological and biochemical status of the animal is outlined in Table 45).

TABLE 45 . DETAILS CONCERNING THE RHESUS MONKEY GIVEN M150 AFTER 56 DAYS

IFA (IgG) against M150	Reciprocal titre 1 : 4096
against LGA 391	" " 1 : 2048
Neut. Ab. against M150	LNI 1.8
against LGA 391	None found
Serum enzyme/substrate (reference Section 3.8.1.6.)	Normal
Serum viraemia	Negative
Temperature	38.1°C
Weight	7.2 Kg

During the 24 day period of study following the second inoculation, the monkey did not develop a fever neither was virus detected in the blood. The serum enzymes and substrates studied (Section 3.8.1.6.) remained normal. The weight of the animal remained stable and there was no detectable pyrexia. The IFA against M150 and LGA 391 remained the same, while the neutralization antibodies continued to rise against M150 to an LNI of 2.8 by day 80. By day 80 LGA 391 neutralizing antibodies could not be demonstrated. Therefore the immunological, biochemical and clinical status of the monkey after a second challenge with M150 is tabulated in Table 46.

TABLE 46. DETAILS CONCERNING THE RHESUS MONKEY GIVEN M 150 (DAY O, DAY 56) AT 80 DAYS POST INFECTION)

IFA (IgG) against M150	Reciprocal titre 1 : 2048
against LGA 391	" " 1 : 1024
Neut. Ab. against M150	LNI 2.8
against LGA 391	None found
Serum enzyme/substrate reference Section 3.8.1.6.)	Normal throughout study
Serum viraemia	Negative
Temperature	38.2°C
Weight	7.4 Kg

3.8.2.6. Re-challenge with LGA 391

The Rhesus monkey which had been given 2 doses of M150 was challenged s.c. after 80 days with 3 $\log_{10}$ $TCID_{50}$ LGA 391 and monitored for 45 days at which time the experiment had to be terminated. The biological status of the animal pre-LGA 391 challenge is outlined in Table 46. A non-immune (M150) Rhesus monkey was used as a positive control.

There was no indication of a febrile illness in the M150 immunised monkey or alteration in IFA values. The serum enzymes and substrate normally affected by Lassa infection (Section 3.8.1.6.) remained normal . No virus was detected in the serum throughout the LGA 391 challenge period . The neutralizing antibody level to M150 remained the same, but low level neutralizing antibody was detected against LGA 391 at day 10 p.i. (LNI 0.3) and increased to an LNI of 1.0 by day 45 post infection. No virus was detected in heart muscle, adrenals, kidneys, liver, spleen, pancreas, small intestine, or lymph nodes after autopsy on day 45 post LGA 391 challenge. Histological examination yielded no obvious lesions or abnormalities which are normally associated with Lassa infection . Therefore the biological status of the monkey after M150 immunisation and LGA 391 challenge is summarised in Table 47).

TABLE 47. DETAILS CONCERNING M150 IMMUNE RHESUS MONKEY CHALLENGED WITH LGA 391 AFTER 45 DAYS

IFA (IgG) against M150	Reciprocal titre 1 : 2048
against LGA 391	" " 1 : 2048
Neut. Ab. against M150	LNI 2.8
against LGA 391	LNI 1.0
Serum enzymes/substrate	Normal throughout study GOT levels increased on day 6 and returned to normal on day 12
" viraemia	Negative
Tissue "	Negative
Histology	Normal

3.8.3. Transmission of other Mopeia viruses to Rhesus monkeys

A series of Rhesus monkeys were given 0.1 ml of tissue culture virus i.p., at either of two dosages, 3 $\log_{10}$ $TCID_{50}$ or 6 $\log_{10}$ $TCID_{50}$. The experimental design is tabulated below in Table 48. The monkeys were monitored over a 28-day period for signs of clinical illness, viraemia, alterations in serum biochemistry, IF and neutralizing antibodies. Samples of blood were taken every two days. Temperatures were taken daily.

TABLE 48 . EXPERIMENTAL INFECTION OF RHESUS MONKEYS WITH MOPEIA VIRUS

Rh. monkey No.	Weight (Kg)	Sex	Mopeia Virus strain	Route of Infection	Virus dose 0.1 ml $TCID_{50}$
3	4.2	Male	M148	i.p.	2
4	3.8	Female	M148	"	6
5	4.2	Female	M150	"	6
6	3.9	Female	M152	"	2
7	4.0	Female	M152	"	6
8	4.5	Male	Z478	"	2
9	4.6	Male	Z478	"	6
10	4.8	Female	None	None	None

3.8.3.1. Clinical observations

None of the monkeys (3-9) became febrile throughout the 28 days and all their temperatures remained within normal limits of 38.6 to 39.8°C. The monkeys remained alert, active and demonstrated no loss of apetite. All animals survived infection with each Mopeia virus at both infective doses.

3.8.3.2. Virus levels in the blood

Virus was not detected in the blood at any time during the study in any of the monkeys.

3.8.3.3. Antibody levels in the blood

The serological responses as measured by IF anti IgG were first detectable between days 10-12 in all monkeys. Peak levels of homologous IF antibody were reached by day 21 in all monkeys (Table 49).

TABLE 49. DEVELOPMENT OF HOMOLOGOUS IMMUNOFLUORESCENT ANTIBODIES OVER 28 DAYS IN RHESUS MONKEYS INFECTED WITH STRAINS OF MOPEIA VIRUS

Rhesus Monkey No.	Mopeia Virus Strain	Dose $\log_{10}TCID_{50}$	Days p.i.					
			9	12	14	19	21	28
3	M148	2	<8	<8	32	1024	4096	4096
4	M148	6	<8	<8	128	1024	4096	4096
5	M150	6	<8	32	4096	4096	8192	8192
6	M152	2	<8	16	512	2048	4096	8192
7	M152	6	<8	32	2048	2048	8192	8192
8	Z478	2	<8	32	4096	4096	8192	8192
9	Z478	6	<8	64	4096	8192	8192	8192

No complement fixing or neutralizing antibodies were detected against homologous Mopeia or heterologous LGA 391 viruses.

3.8.3.4. Clinical biochemistry

There was no alteration in the GOT, GPT, LDH, αHBDH, γGT, CK, LAH, urea, total protein, triglycerides, creatinine or cholesterol levels in any of the monkeys throughout the 28 days.

3.8.4. Challenge of Mopeia-immunised Rhesus monkeys with LGA 391

The Rhesus monkeys (3-9) that had been infected with various strains of Mopeia virus were challenged on day 60 post Mopeia infection, with LGA 391. They were challenged s.c. with 0.1 ml virus tissue culture fluid, the final dose being 3 $\log_{10}$ $TCID_{50}$. A control non-immunised Rhesus monkey (10) was included and acted as a positive LGA 391 control. The monkeys were monitored over a 28-day post Lassa challenge period. Samples of blood were taken every two days for virological, immunological and biochemical analysis. Temperatures were taken daily. Baseline details on each Rhesus monkey are outlined in Table 50.

TABLE 50. DETAILS CONCERNING RHESUS MONKEYS 60 DAYS AFTER MOPEIA IMMUNISATION AND PRIOR TO CHALLENGE WITH LGA 391

RH monkey No.	3	4	5	6	7	8	9	10
Immunising Virus	M148	M148	M150	M152	M152	Z478	Z478	None
Homologous IFA*	4096	4096	4096	2048	2048	1012	2048	<8
Heterologous IFA* against LGA 391	2048	1024	1024	1024	1024	1024	1012	<8
Homologous Neut. Ab.**	ND	1.0	1.8	1.0	ND	1.6	1.8	ve
Heterologous Neut.Ab. against LGA 391	-ve	-ve	-ve	-ve	-ve	-ve	-ve	ve
Serum enzyme & substrates	N	N	N	N	N	N	N	N
Viraemia	-ve-	-ve	-ve	-ve	-ve	-ve	-ve	ve
Temperature (°C)	39.2	39.2	39.3	39.5	39.6	39.3	39.1	38.7
Weight (Kg)	4.8	4.0	4.3	4.2	4.0	4.6	4.7	3.8

* reciprocal antibody titres

** expressed as LNI

N - Normal N.D. not done

3.8.4.1. Clinical observations

None of the immunised Rhesus monkeys became febrile throughout the 28 days p.i. and all the temperatures remained within normal limits, 38.6 - 39.8°C. The control monkey demonstrated a febrile illness which started to rise above normal on day 6 (40°C) and reached a peak on day 9 (41°C). Between days 10-12 the temperature of the control monkey gradually decreased to sub-optimal levels (36.7°C) at which time it became moribund. A summary of the clinical observations is made in Table 51).

TABLE 51. COMPARATIVE SUMMARY OF CLINICAL OBSERVATIONS FROM MOPEIA IMMUNE RHESUS MONKEYS AFTER CHALLENGE WITH LGA 391

Rhesus monkey No.	3	4	5	6	7	8	9	10
Immunising Virus	M148	M148	M150	M152	M152	Z478	Z478	None
Dose $Log_{10}TCID_{50}$	2	6	6	2	6	2	6	-
Febrile illness	No	No	No	No	No	No	No	Yes
Food and H_20 intake	N*	N	N	N	N	N	N	Reduced to nil
Activity	"	"	"	"	"	"	"	Reduced from day 8
Weight reduction	None	None	None	None	None	None	None	240g
Outcome of challenge	S**	S	S	S	S	S	S	Moribund day 12

*N - Normal

** - Survived

3.8.4.2. Virus levels in blood

No LGA 391 was found in any of the blood samples taken from any of the Mopeia Rhesus monkeys, throughout the 28 days post Lassa challenge. In comparison, LGA 391 was isolated from the control Lassa Rhesus monkey from day 6 and remained in evidence until the animal became moribund on day 12 (Table 52 and Fig.89).

TABLE 52. COMPARATIVE VIRAEMIA OBTAINED FROM RHESUS MONKEYS IMMUNISED WITH STRAINS OF MOPEIA AND CHALLENGED WITH LGA 391

	Serum virus titre ($Log_{10}TCID_{50}$/ml) at various days										
	2	4	6	8	10	12	14	16	18	21	28
Rhesus monkey Nos. (3-9)	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	ve
Control LGA391 Rhesus monkey (10)	-ve	-ve	2.7	6.3	6.3	5.8 Died					

3.8.4.3. Clinical biochemistry

In the case of the control Rhesus monkey challenged with LGA 391, the serum enzymes GOT, GPT, LDH, αHBDH. CK and AP gradually increased above normal to their maximum by day 12 when the animal became moribund (Fig. 90).

The serum biochemistry of the immunised monkeys after challenge demonstrated slight alterations in the GOT levels. The GOT values rose above normal levels on day 6 in those monkeys immunised with only 2 $log_{10}TCID_{50}$ Mopeia viruses (M148, M152, Z478) and challenged with LGA 391 (Figs. 91 and 92). The levels varied between 3-4 times normal and were consistent with each monkey immunised with the Mopeia strains. The GOT levels in each case returned to normal by day 12. The rise in the GOT level was not evident in those monkeys immunised with 6 $log_{10}TCID_{50}$ Mopeia strains (M148, M150, M152 and Z478). The other serum enzymes studied (LDH, αHBDH, γGT, CK, SD and AP) all remained normal throughout the 28 days. The serum substrates (triglycerides, total proteins and urine) also remained within normal limits throughout the 28 days.

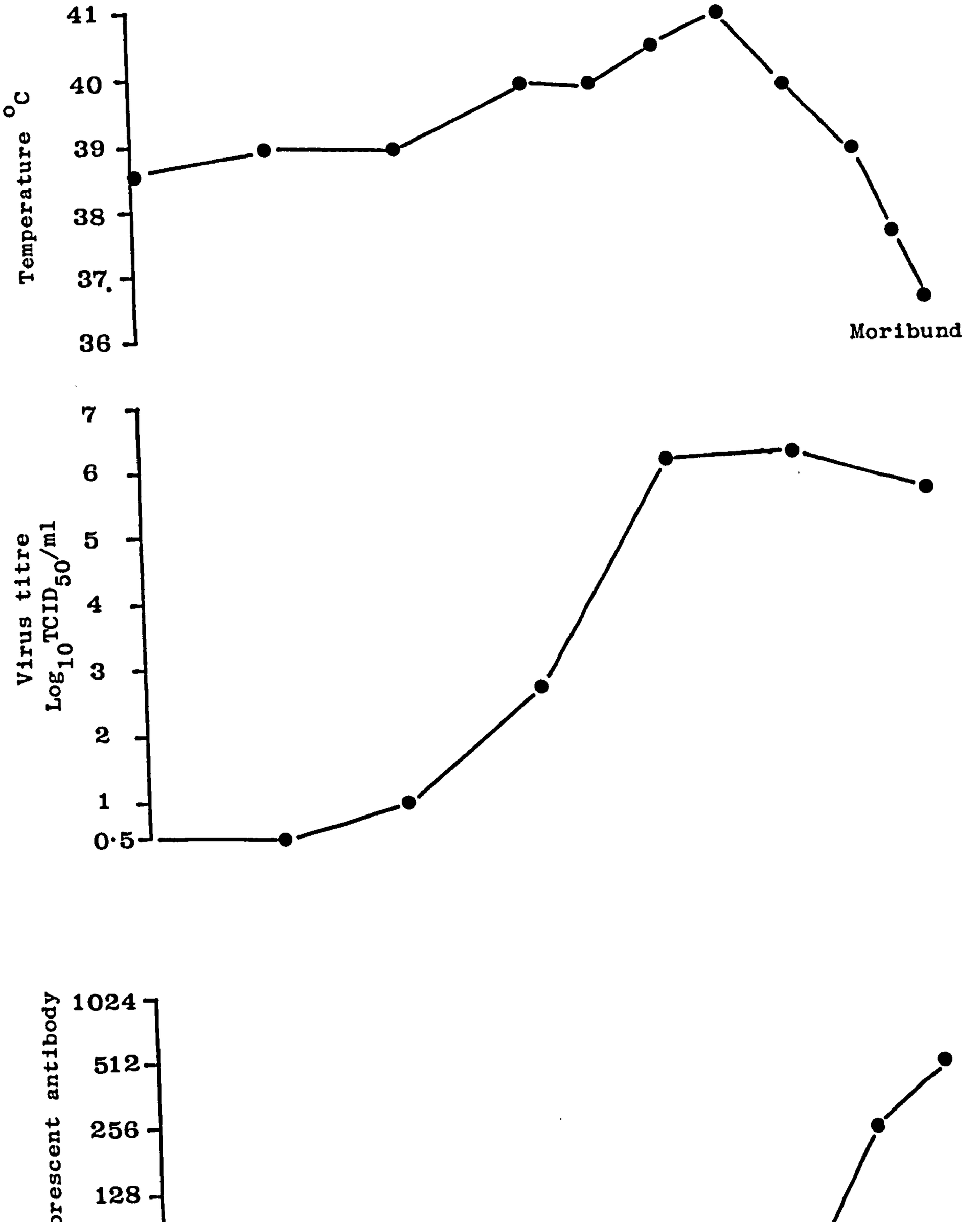


Fig.89 Course of Lassa fever in a Rhesus monkey inoculated with 3 $\log_{10}TCID_{50}$ by the s.c. route

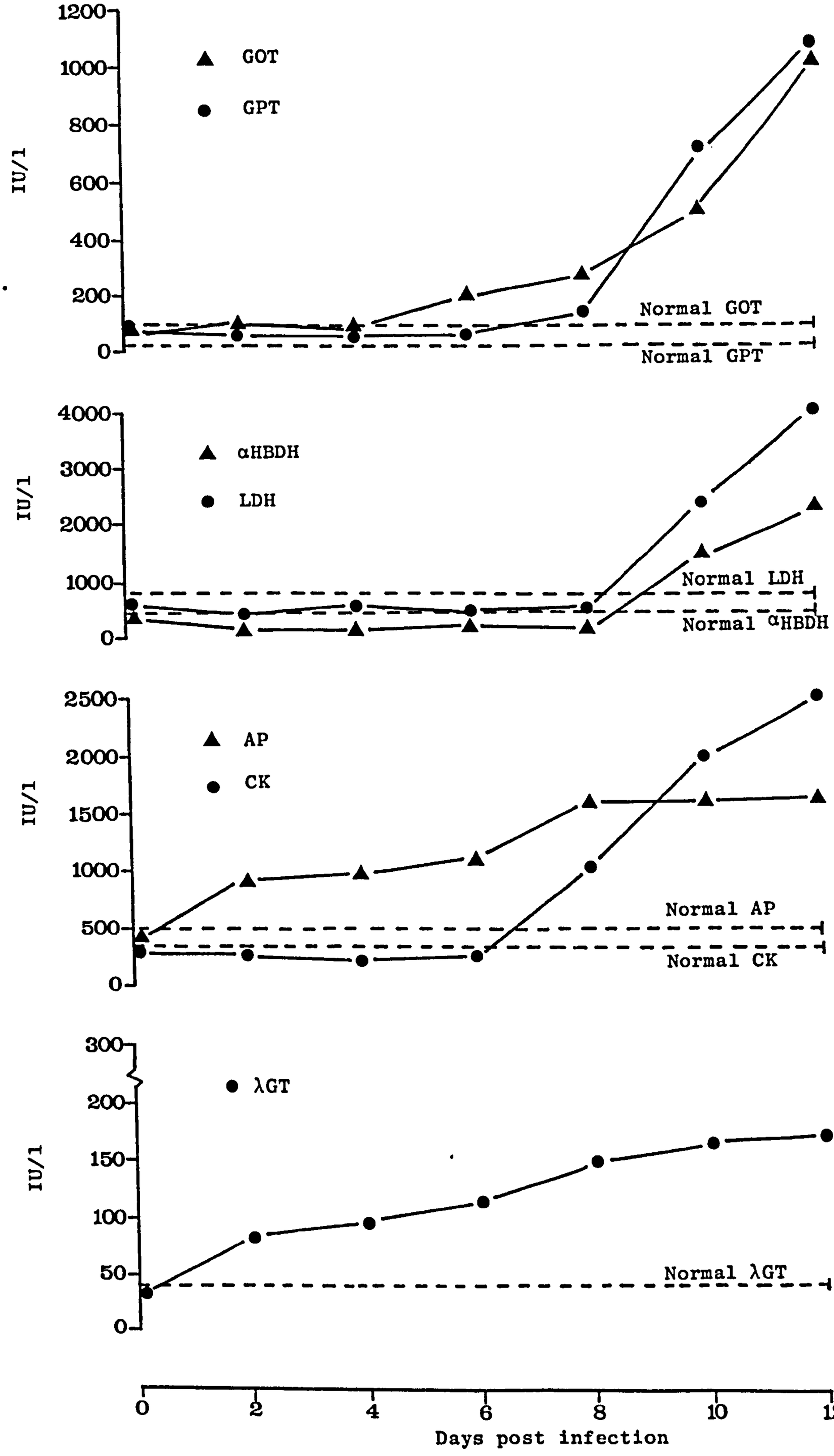


Fig.90 Activity of various enzymes in the sera of Rhesus monkeys after s.c. infection with 3 $\log_{10}$ $TCID_{50}$ LGA 391

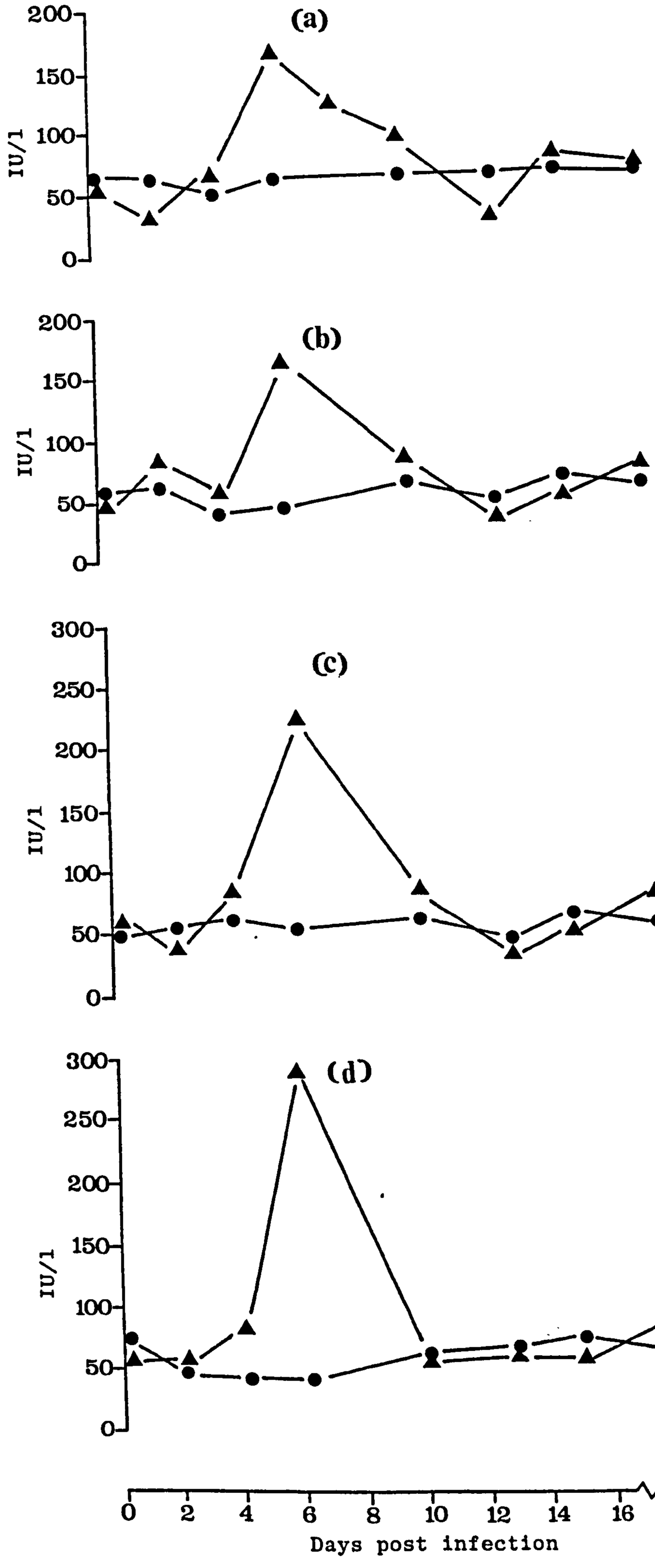


Fig.91 Glutamine oxaloacetate (GOT) and Glutamine pyruvate transaminase (GPT) activity in the serum of Mopeia immunised (2 $\log_{10}TCID_{50}$) Rhesus monkeys after challenge with LGA 391 (3 $\log_{10}TCID_{50}$). (a) M152, (b) Z478, (c)M150, (d) M148.

● GOT ▲ GPT

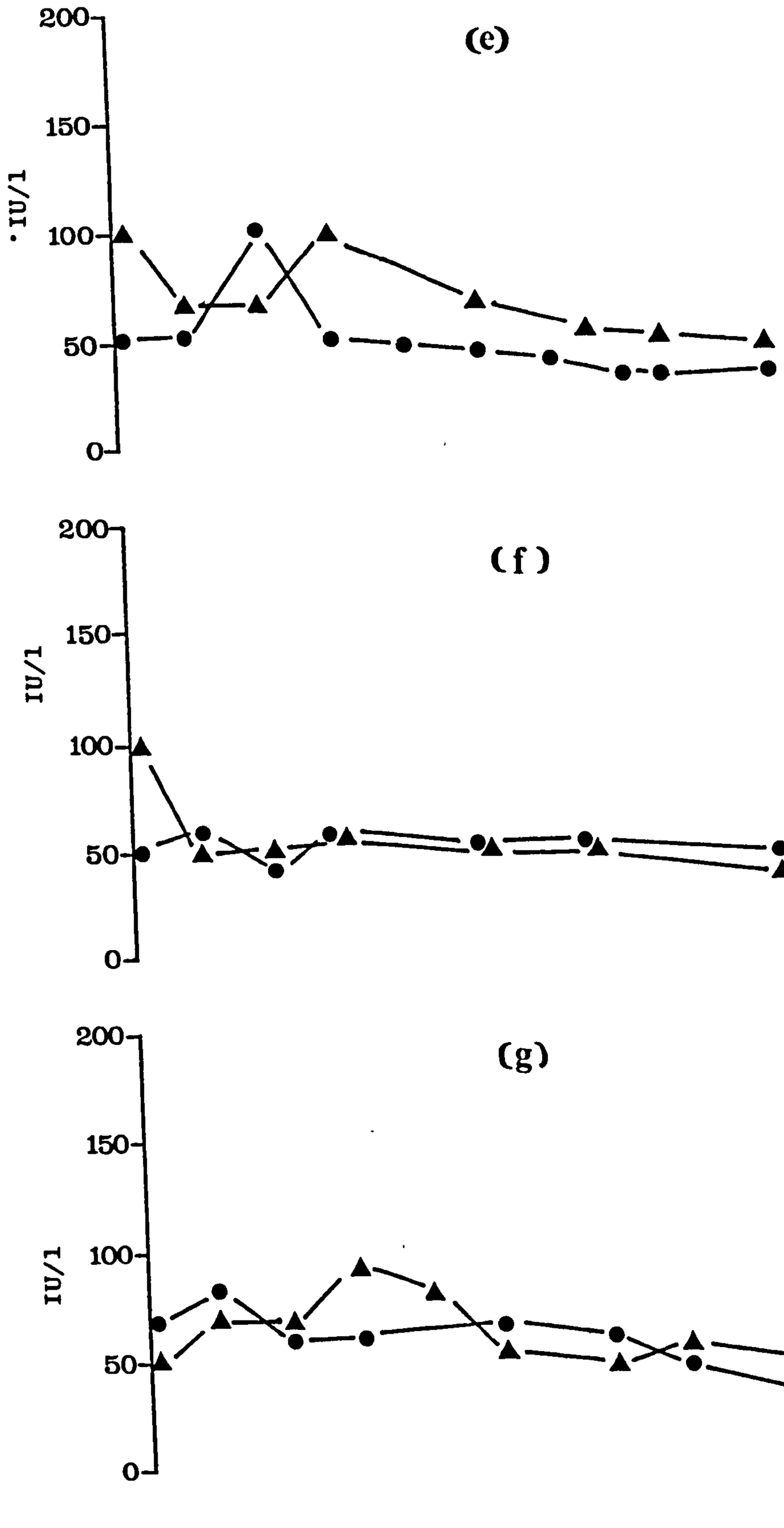


Fig.92 Glutamine oxaloacetate (GOT) and Glutamine pyruvate transaminase (GPT) activity in the serum of Mopeia immunised (6 $\log_{10}TCID_{50}$) Rhesus monkeys after challenge with LGA 391 (3 $\log_{10}TCID_{50}$). (e) M152, (f) M148, (g) Z478.

● GOT ▲ GPT

3.8.4.4. Antibody levels in the blood

The reciprocal IF homologous antibody levels of Mopeia immunised Rhesus monkeys after LGA 391 challenge are shown in Table 53. IF values are reduced between days 2-8 post challenge, thereafter rising to slightly higher levels by day 28. The anti-LGA 391 IF antibodies demonstrated the same fluctuations.

TABLE 53. RECIPROCAL IgG IF HOMOLOGOUS ANTIBODY LEVELS IN MOPEIA IMMUNISED MONKEYS OVER 28 DAYS POST LGA 391 CHALLENGE

Rhesus Monkey No.	Immunising virus(Dose $TCID_{50}$)	Day post LGA 391 challenge 0	2	4	6	8	10	12	14	21	28
3	M148(2)	1024	512	128	512	256	512	1024	1024	2048	4096
4	M148(6)	2048	1024	512	2048	ND	ND	4096	4096	4096	4096
5	M150(6)	2048	1024	2048	2048	ND	ND	4096	4096	4096	4096
6	M152(2)	2048	2048	512	512	512	ND	2048	ND	2048	2048
7	M152(6)	1024	512	1024	1024	512	ND	2048	ND	2048	2048
8	Z478(2)	1024	1024	1024	1024	512	ND	1024	4096	ND	4096
9	Z478(6)	2048	1024	512	256	256	ND	1024	2048	4096	4096
10	-ve	<8	<8	<8	8	16	256	512	Died		

The appearance of neutralizing antibodies in all the monkey sera demonstrated varying levels. The anti-LGA 391 activity as measured by LNI did not rise above 0.5 in any of the Mopeia challenged monkeys 28 days post Lassa challenge. The LNI levels are shown in Table 54.

TABLE 54. HOMOLOGOUS AND HETERLOGOUS NEUTRALIZING ANTIBODIES IN THE SERA OF MOPEIA IMMUNISED RHESUS MONKEYS 28 DAYS POST LGA 391 CHALLENGE

Rh. Monkey No.	Immunizing Virus and (Dose)	LNI	
		Homologous	Heterologous (anti-LGA 391)
3	M148(2)	1.5	-ve
4	M148(6)	2.0	0.3
5	M150(6)	2.0	0.5
6	M152(2)	1.5	0.3
7	M152(6)	2.1	0.5
8	Z478(2)	1.8	0.5
9	Z478(6)	2.0	0.5

3.9. STUDIES ON A HUMAN CASE OF LASSA FEVER

In the light of studying the effects of LGA 391 and Mopeia virus strains in Rhesus monkeys, an ideal opportunity arose during the course of the work to study a human case in the acute and convalescent stages of the disease and to directly compare the results with those found in the Rhesus monkey.

3.9.1. Case history

An 18-year-old Nigerian woman visitor (P.B.)to London fell ill on 28 December 1981 with fever, headache, myalgia, nausea and vomiting (Fig. 93). By 4 January 1982, she was so unwell that she was referred to St Thomas' Hospital. By 8 January, her condition was serious and a diagnosis of Lassa fever seemed to be a very strong possibility. In view of this and the possible risk of infection in the event of haemorrhage, she was transferred to the high security infectious disease unit at Coppetts Wood Hospital, where she was admitted into a Trexler negative pressure patient isolator.

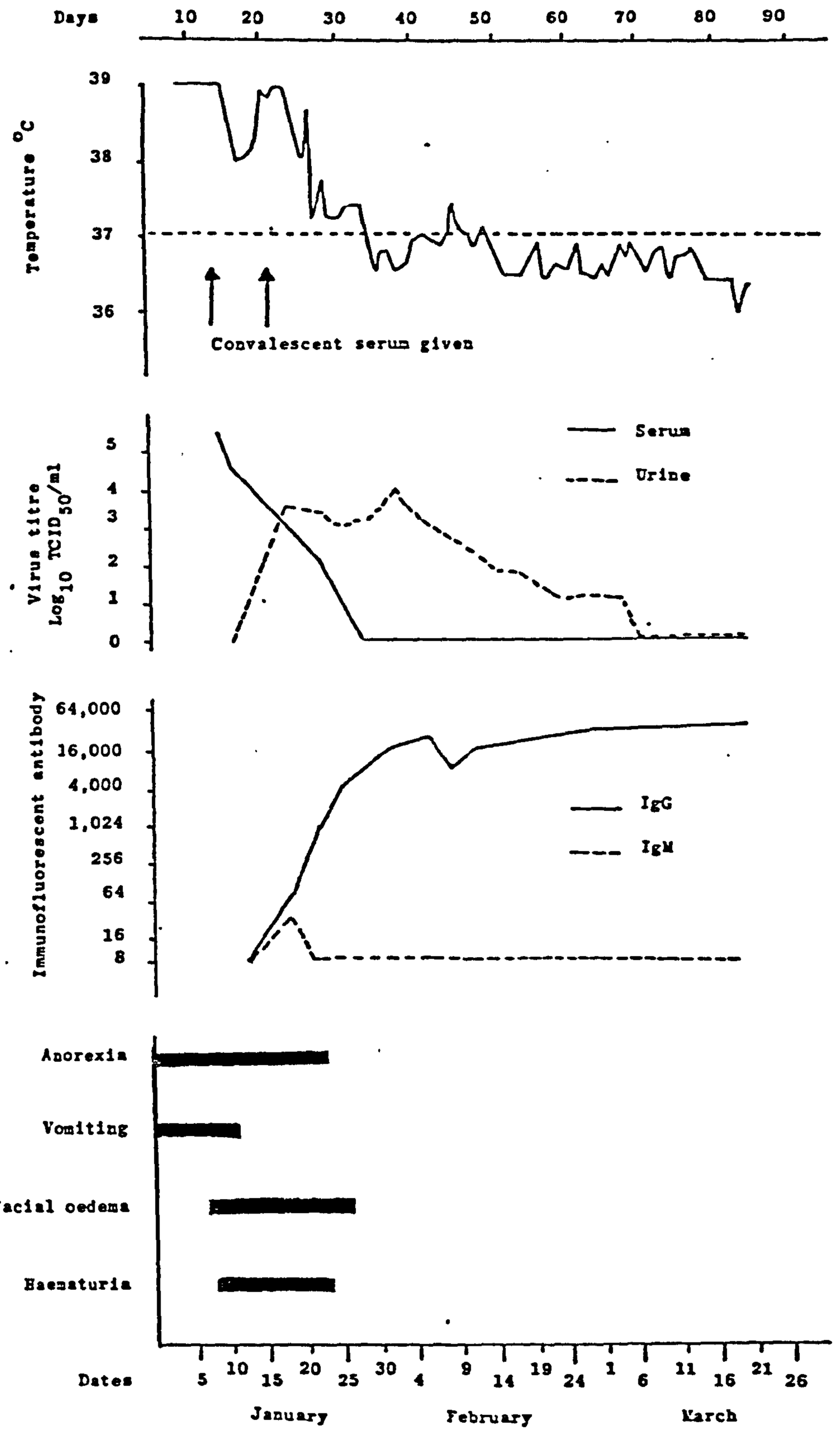


Fig.93 Course of Lassa fever in patient (P.B.)

3.9.2. Virological and Serological studies

The first specimen of blood collected for virological studies on 8 January 1982, 12 days after the patient became feverish, was examined by electron microscopy and arenavirus particles were seen. Tissue culture which had been inoculated with the blood proved positive by IFA 2-3 days later.

Virus isolation and serological studies were made on specimens of blood, urine and throat washings collected during the acute and convalescent phases of the illness. The highest level of virus in the blood ($10^{5.2}TCID_{50}$/ml) was found in the sample taken on 8 January 1982; virus declined to undetectable levels by day 32 (21 January 1982) after onset of illness (Fig. 93).

No virus was isolated from urine samples collected before day 22 (18 January 1982). Thereafter virus was evident until day 67. The peak level of virus ($10^{4.0}TCID_{50}$/ml) was found in the urine on day 36 but levels ranged from $10^{2.0}$-$10^{4.0}TCID_{50}$/ml between days 22 to 53. Undetectable virus was observed by day 71.

All throat washings taken throughout the patient's isolation yielded no virus.

Circulating antibody levels as determined by IFA demonstrated an anti-Lassa IgG antibody of 1 : 64 present on day 22 and peak levels of 1 : 24,000 and 1 : 32,000 being reached on days 44-55. These antibody levels were sustained throughout her convalescence. Circulating anti-Lassa IgM antibody of 1 : 16 - 1 : 32 were detectable between day 19 and day 21 only.

Convalescent sera given on day 12 and day 17 had an IFA level of 1 : 1024 and 1 : 2048 respectively but no CF or neutralizing antibodies were detectable.

A CF antibody level of 1 : 16 was observed on day 50 which rose to 1 : 64 on day 76. Anti-Lassa GA 391 neutralization antibodies of 1 : 256

were observed on day 56 and 1 : 64 on day 76. No neutralising antibodies were evident before day 56.

3.9.3. Clinical Biochemistry

Normal values of human serum enzymes and substrates were determined using random human serum on the LKB Analyser. A comparison was made using this data with the results obtained from the serum obtained from P.B.

TABLE 55. NORMAL VALUES OF SERUM ENZYMES AND SUBSTRATES IN MAN

Enzymes/Substrates	Normal Values		Units
	Men	Women	
GOT	40	35	IU/l
GPT	45	35	"
LDH	450		"
α-HBDH	125		"
CK	24 - 170	24 - 150	"
γGT	11 - 50	7 - 30	"
AP	270		"
Creatine	3 - 97	44 - 80	mg/100 ml
Urea	1.7 - 8.3		"
Total protein	6.6 - 8.7		"
Triglycerides	70 - 170		"
Cholesterol	7.5 - 6.7		"

In the acute phase of her disease, various enzyme levels rose progressively. By day 7 after onset the GOT rose to a peak level of 4440 IU/l (normal 7-20 IU/l) and GPT 850 IU/l (normal 8-30 IU/l) (Fig. 94). Both these enzymes in the serum were reduced to normal levels by day 18 after onset of illness.

Lactate dehydrogenase (LDH) and hydroxybutyrate dehydrogenase (HBDH) (Fig.94) were also at the highest levels at day 7 after onset of illness at 4003 IU/l and 4015 IU/l respectively. The LDH and HBDH were reduced to normal levels by day 32.

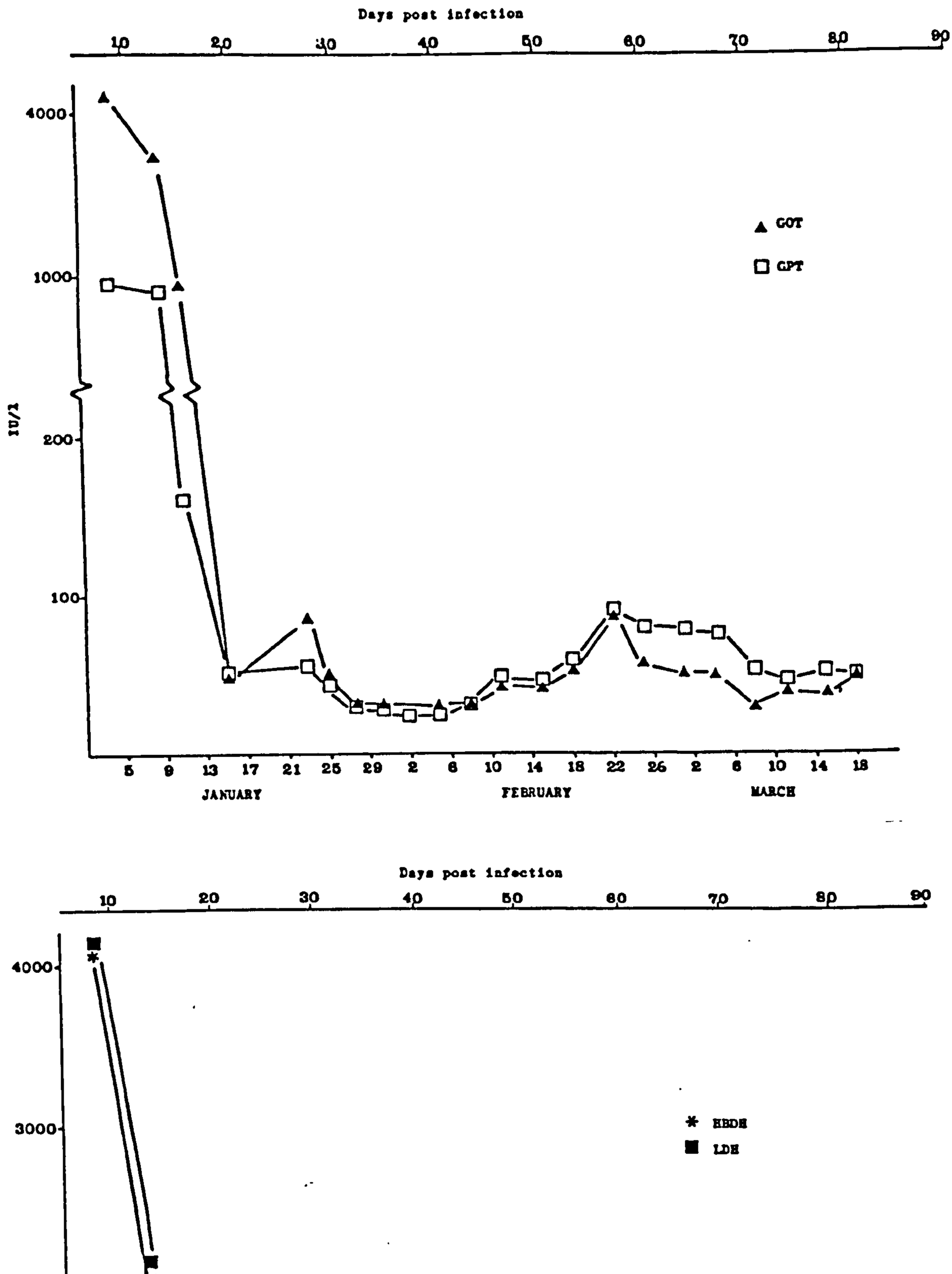


Fig.94 Demonstration of serum enzyme levels from a Lassa patient (P.B.) during the course of the illness

Creatine kinase (CK) (Fig. 95) levels 7 days after onset were raised to 123 IU/l (normal <65 IU/l), and decreased to normal levels by day 14. The CK activity increased to its peak level of 312 IU/l on day 19 and decreased to normal levels by day 21. The levels remained at approximately twice their normal values during convalescence.

LAH (Fig. 95) was also at its peak level during the acute phase of the disease. On day 7 the value of 250 IU/l was observed which decreased to normal by day 19 and remained normal throughout the course of the disease.

AP (Fig. 95) reduced from its peak level of 225 IU/l on day to 120 IU/l on day 76.

γGT (Fig. 96) remained high during the acute phase of the disease at 60-80 IU/l (day 7-22) falling to normal levels during the convalescent phase.

Urea levels remained above normal throughout the course of the infection even in the convalescent phase (Fig. 96).

Creatine and Total Proteins were already high on admission to hospital (day 7) but returned to normal throughout the course of the infection. Cholesterol and triglyceride values fluctuated throughout the illness.

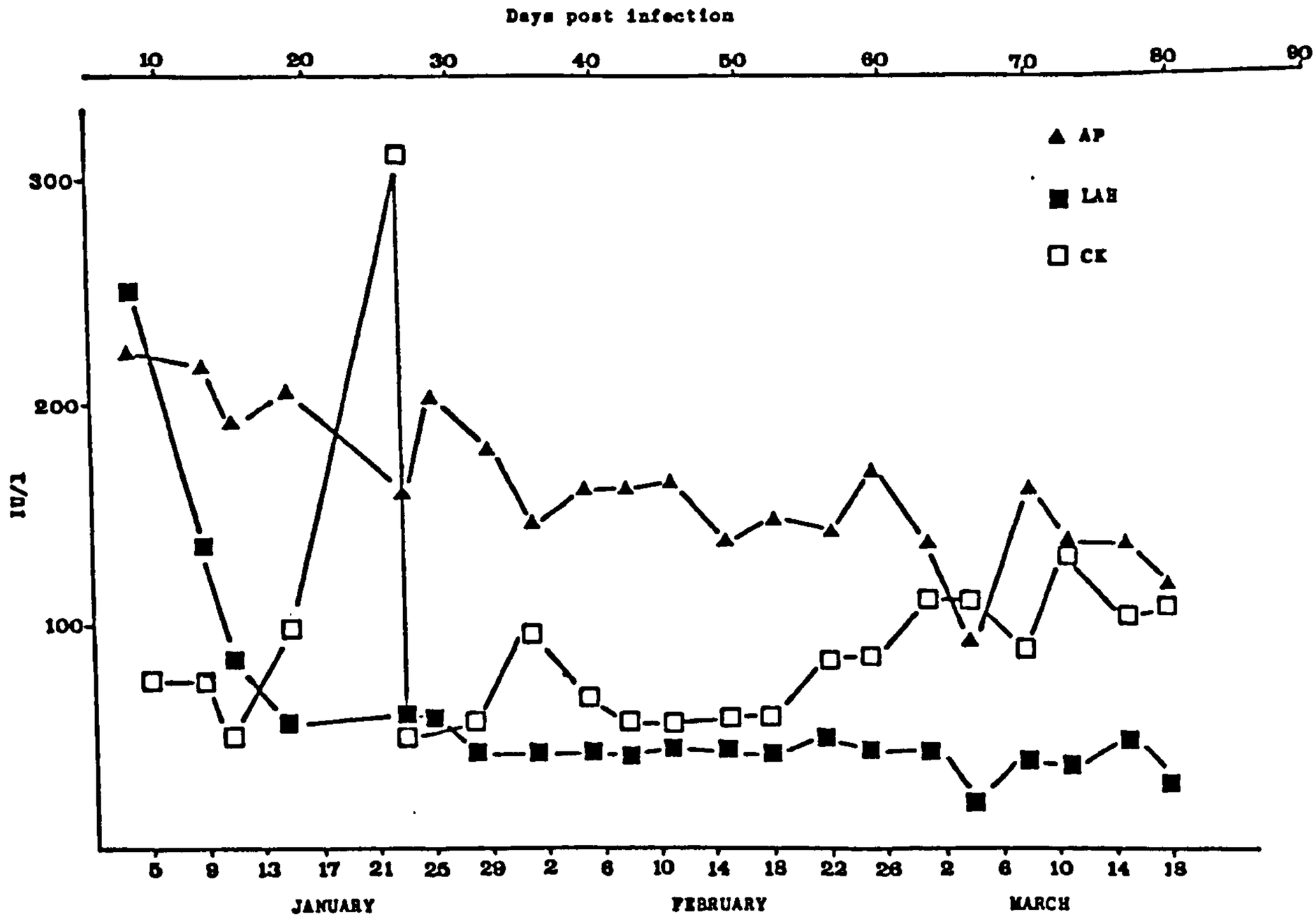


Fig.95 Demonstration of serum enzyme levels from a Lassa patient (P.B.) during the course of the illness

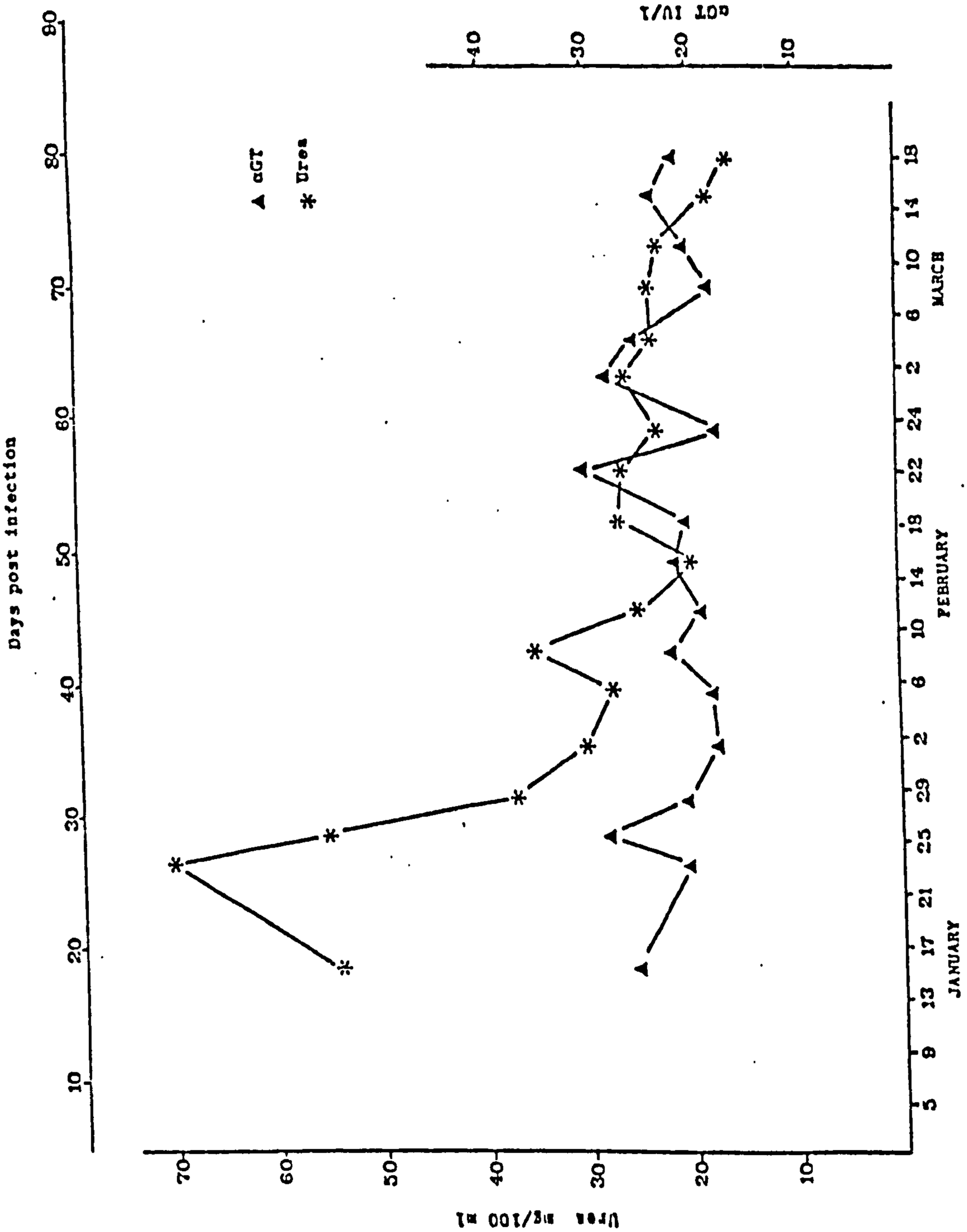


Fig.96 Demonstration of serum enzyme and substrate levels from a Lassa patient (P.B.) during the course of the illness.

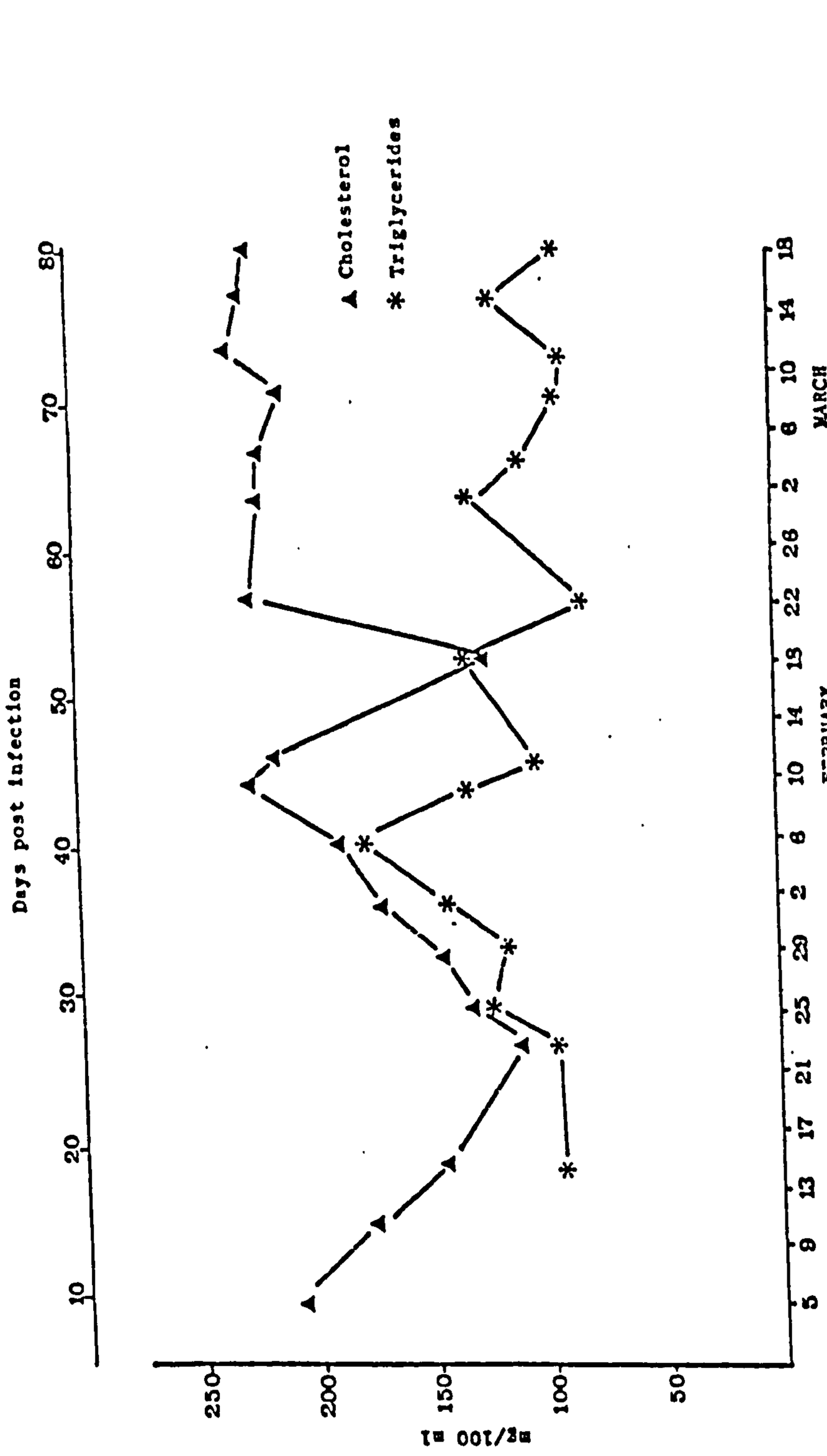


Fig.97 Demonstration of serum enzyme and substrate levels from a Lassa patient (P.B.) during the course of the illness.

4. DISCUSSION

The International Committee on Taxonomy of Viruses (ICTV), having accepted the recommendation of a Study Group (Pfau et al., 1974), proposed that the name 'Arenaviridae' be given to a set of viruses previously designated arenaviruses. Their most striking characteristic is a unique morphology and additionally all the component viruses are antigenically related to a greater or lesser extent, depending on the serological assay employed and the potency of the antigens and antisera. The implications of molecular biological comparisons, the use of monoclonal probes, of shared antigens on specific diagnosis, protection against cross-challenges and immunopathological developments resulting from sequential exposures to different viruses are also to be borne in mind.

LCM virus has been chosen as the prototype virus for the family in which there are at present 12 members (Pedersen, 1979; Wulff et al., 1978). The group is divided into the LCM complex ('Old World') and the Tacaribe complex ('New World') on the basis of serological cross-reactivity demonstrated by CF or IF assays. Besides LCM, the other members of the 'Old World' group are Lassa and Mopeia viruses. Lassa virus, like most other arenaviruses, appears to be a single-host rodent parasite, and because M. natalensis is widely distributed over the African continent (Isaacson, 1975), the presence of a virus (Mopeia) closely related to Lassa does not represent a biological surprise, especially when one compares it with the distribution of arenaviruses in South America. Mopeia viruses, in contrast, while isolated from the same rodent species, are found in Mozambique (Wulff et al., 1977), and Zimbabwe (Johnson et al., 1981), where a disease like Lassa fever has not been reported. In addition, a Lassa related virus has been isolated from Praomys sp. found in the Central African Republic (CAR) which is considered to be distinct from the other African arenaviruses (Gonzalez, et al. 1983). The Mopeia and CAR isolates raise

several questions of more than academic interest. Are the viruses a minor antigenic variant of Lassa virus or are they new arenavirus immunotypes? It is important to determine if the new strains are pathogenic for man and whether M. natalensis from West Africa and south-east Africa are truly identical. The question of pathogenicity will only be answered on the chance recognition of Lassa fever outside West Africa or the elucidation of pathogenic properties and their comparative biology of Lassa and Mopeia viruses in animals such as a non-human primate. Therefore, within the context of the stated hypothesis of this thesis (Section 1, page 14) the characteristics of the Mopeia viruses (Tables 56, 57 and 58) will be examined and compared with the established view of the arenavirus family.

Isolation of arenaviruses in vitro does not readily distinguish various strains. Lassa and Mopeia viruses grow readily in Vero and CV-1 cell lines producing an obvious cytopathic effect. In the present studies a cytopathic effect developed within 3-4 days post inoculation and was very obvious by day 5 in the case of Lassa and by day 7 with all Mopeia viruses. Lassa was the most productive virus in Vero or CV-1 cells reaching levels 10-100 fold higher than that produced by the Mopeia viruses. In stock pools of Lassa and Mopeia viruses with titres $>10^6$ no cell destruction was observed in assay systems until the 10^{-3} dilution. This phenomenon of autointerference has been previously reported for LCM and other arenaviruses (Welsh et al., 1975; Dutko et al., 1976). This interference was observed shortly before maximal infectious virus synthesis and increased as the age of the culture increased. Welsh and Pfau (1972) demonstrated with LCM infection of BHK-21 cells, an interfering component which inhibited LCM synthesis. Dilution of the inoculum before infection reduced the interference and resulted in 100-fold more infectious virus, a similar increase being observed with both Lassa and all the Mopeia viruses in the studies carried out in this Thesis. On the basis of

several physicochemical studies, Welsh and Pfau (1972) suggested that the interfering component was a defective interfering (DI) virus. A number of studies indicated that LCM produces DI virus (reviewed by Huang, 1973) and that these may play a role in persistent infections. DI virus was first separated from infectious LCM in the culture fluid from BHK-21/13s cells persistently infected with LCM (Welsh et al., 1972; Staneck et al., 1972). It is now clear that LCM DI (i) sediments to a lower density in sucrose than LCMV (Oldstone et al., 1977; Welsh and Buchmeier, 1979), (ii) contains infectious LCM structural proteins (Welsh and Buchmeier, 1979), (iii) does not reproduce itself in culture unless infectious LCMV is also present, (iv) interferes specifically with LCMV but not heterologous viruses (Welsh and Pfau, 1972), and (v) has a target size for UV inactivation significantly less than infectious LCMV suggesting a reduced genome size (Welsh et al., 1972; Popescu et al., 1976). Attempts to isolate or prove the existence of DI particles with Lassa or Mopeia cultures proved unsuccessful even though the interference phenomenon in those cultures was observed.

In addition it has been shown that some tissue culture types infected with LCM, show no CPE over extended periods but still retain infective virus (Hotchin and Cinits, 1956). Similar observations were made with cultures of Tamiami (Calisher et al., 1970), Junin (Coto et al., 1974), Parana (Staneck et al., 1972), Pichinde (Dutko and Pfau, 1978), and Lassa viruses (Buckley and Casals, 1970). The studies with Lassa and all the Mopeia strains in infected L cells show the same arenavirus characteristics.

4.1. Physiochemical properties (Table 56)

The similarities in morphology and morphogenesis of Lassa and Mopeia viruses are so marked and distinctive that they all conform to the basic electron microscopical pattern attributed to the arenaviridae (Figs. 23-42). Thin section electron microscopy of Vero, CV-1 or L cells infected

TABLE 56. SUMMARY OF THE PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES COMMON TO LASSA LGA 391 AND MOPEIA VIRUSES

Properties	Virus		
	LGA 391	Mopeia viruses	
Origin	Nigeria	Mozambique	Zimbabwe
RNA genome	+	+	+
Infectious nucleic acid	-	-	-
Overall shape spherical to pleomorphic	+	+	+
Diameter nm	80-250	80-240	120-130
Surface projections club shaped, 10 nm long	+	+	+
Contains host ribosomes	+	+	+
Replicating potential	+	+	+
BUDR sensitivity	-	-	-
Rapidly inactivated between pH 5.5 and above 8.5	+	+	+
UV sensitive	+	+	+
Heat sensitive 56°C, 60°C	+	+	+
Lipid solvent sensitive	+	+	+
BPL, formalin, glutaraldehyde sensitive	+	+	+
Immunizing antigen	$(\underline{+})^{*}$	+	+
Interference potential	+	+	+
Cytoplasmic effect	+	+	+
Cytoplasmic antigen	+	+	+
Cell surface antigen	+	+	+

* observed in experimental animals that survived LGA 391 infection.

+ = present - = absent + = equivocal

with LGA 391 or Mopeia viruses show them to be indistinguishable from each other. The particles are round, oval or pleomorphic, 60-280 nm in diameter, having a membraneous envelope with surface projections or spike approximately 6-10 nm long, and contain a variable number of internal electron-dense granules about 20 nm in diameter which strongly resemble ribosomes. Comparison between LGA 391 and Mopeia viruses show that there is a striking morphological similarity with LCM, Machupo and Tacaribe (Murphy et al., 1969), Latino, Parana and Tamiami (Calisher et al., 1970). The envelope of the LGA 391 and Mopeia virus is consistent with the other arenaviruses in that it consists of two electron-dense layers supported by a broader electron-lucent intermediary zone (Murphy and Whitfield, 1975). The virus particles are formed by a process of budding at the cell surface. During this process there is continuity of the unit membrane of the cell surface and the viral envelope. As with all arenaviruses, development of the viral envelope is denser and more distinct than that of the host membrane from which it is derived, a feature shown with LGA 391 and Mopeia viruses. Late in infection large areas of plasma membrane become dense, seem to have virus surface projections and may accumulate a layer of amorphous material in patches.

Negative-contrast electron microscopy of virus particles sedimented from infected cell cultures demonstrates uniformity of the viruses, with pleomorphic particles slightly larger than in thin sections, 90-350 nm, pronounced surface projections, and no resolution of internal structure (Murphy et al., 1970).

Electron microscopy of animal tissue proved unproductive in the case of Lassa and Mopeia viruses. In general, only occasional virus particles were observed in the brain tissue of mice infected with LGA 391 and Mopeia strains, which with parallel studies using CFT and IF indicated the

presence of specific antigen. No particles were found in tissues of guinea pigs or monkeys infected with Mopeia viruses, a situation not uncommon with that found with Tacaribe and Junin viruses (Casals 1975).

The common characteristics of viral morphology and development link Mopeia viruses to members of the arenaviridae. In addition, the physicochemical characteristics studied and outlined in Table 56 demonstrate further compatibility with the arenavirus family. All arenaviruses so far studied including Mopeia and LGA 391 viruses are sensitive to lipid solvents such as sodium desoxycholate (Buckley and Casals, 1970; Calisher et al., 1970; Downs et al., 1963; Mettler et al., 1961; Trapido and Sanmartin 1971), chloroform (Johnson et al., 1965; Webb et al., 1970) and ether (Mifune et al., 1971). Similarly a rapid loss in infectivity below pH 5.5 and above pH 8.5 was a consistent finding in the present study and with other arenaviruses like Pichinde (Mifune et al., 1971), Junin (Parodi et al., 1966) and Parana (Webb et al.,1970). They are relatively heat-sensitive, infectivity half-lives being a matter of minutes at 60°C and 56°C, hours at 37°C and days at 25°C and 4°C (Calisher et al., 1970; Lloyd et al., 1982; Mifune et al., 1971; Parodi et al., 1966; Webb et al., 1967). It is impossible to make exact comparisons here because no experiments have been done with purified virus and/or under identical conditions. Although no direct inactivation data are available, human sera containing Lassa virus remained infectious after 4 days at crushed ice temperature while in transit from West Africa to New York (Buckley and Casals, 1970). It is important to note the temperature sensitivity of the arenaviruses especially with Lassa and to a certain extent Mopeia, since treatment of purified or tissue culture extracts at 60°C under high containment should provide a means of safely removing the samples for analytical study in less restrained conditions, i.e. molecular biological analysis, providing they do not interfere with the analysis being carried out or the final results.

No DNA synthesis-inhibiting compound has been found to inhibit arenavirus replication. LCM synthesis was unaffected by concentrations of halogenated deoxyuridines (BUDR, FUDR, IUDR) that inhibits DNA containing viruses (Hotchin, 1971; Lehmann-Grube, 1971). The Mopeia and LGA 391 viruses in the present study plus Lassa (Buckley and Casals, 1970), Junin (Martinez Segovia and Grazioli, 1969), Machupo (Webb et al., 1967) and Parana (Webb et al., 1970) were all resistant to BUDR. Definitive proof of the RNA content of arenaviruses was obtained after chemical analysis of purified LCM and Pichinde viruses (Pedersen 1970; Coto, 1973). The arenaviruses contain a single-stranded RNA genome of total molecular weight of about 4×10^6 (Ramsingh, 1980) in two segments of negative polarity.

The protein structure of purified Lassa virus (Clegg and Lloyd, 1982; Kiley et al., 1981) is very similar to that of other arenaviruses which have been analysed. The major component of the virus particle is the nucleocapsid protein with a molecular weight of 60,000 compared with 54,000-68,000 reported for other arenaviruses (Pedersen, 1979; Howard and Simpson, 1980). Lassa and Mopeia viruses (Kiley et al., 1981; Clegg and Lloyd, in preparation) also have two envelope glycoproteins with molecular weights similar to those found with other members of the family, although Tacaribe and Tamiami viruses are reported to have only a single envelope glycoprotein (Gard 1977; Boersma et al., 1982). There are also some similarities between the minor virion components of Lassa and Mopeia viruses (Clegg unpublished) and those found in other arenaviruses. In addition an RNA polymerase activity, which would be expected in a negative strand RNA virus has been found in purified preparations of Pichinde virus (Carter et al., 1974; Leung et al., 1979) as well as a protein kinase activity in purified LCM (Howard and Buchmeier, 1983). These enzyme activities have not yet been identified with polypeptides

observable by polyacrylamide gel electrophoresis. A further virus-specific protein (GPC) has been found in immunoprecipitates from cells infected with LCM, Tacaribe or Pichinde viruses (Buchmeier et al., 1978; Buchmeier and Oldstone, 1979; Buchmeier et al. 1980b; Harnish et al., 1981; Saleh et al., 1979). This is a glycoprotein with a molecular weight of 70,000-79,000 which appears to be processed by proteolytic cleavage and carbohydrate modification to give the mature envelope glycoproteins. Analysis of purified LGA 391 has shown a major protein component very similar to that of other arenaviruses (Clegg and Lloyd 1982).

The N protein of arenaviruses is similar in size and proportion to the other structural proteins. This homogeneity does not extend to glycoproteins. The common major glycoprotein species is the GP-2 protein (33-44,000 m.w.) found in all examined arenaviruses including Mopeia virus (Clegg and Lloyd, unpublished). The other glycoprotein (GP-1 (52-65,000 m.w.) is more variable in size. The GP-1 and GP-2 glycoproteins are thought to be located at the surface of the virus. This conclusion is based on work treating Pichinde and LCM viruses with pronase and bromelain (Vezza et al., 1977; Buchmeier et al., 1978) or Tacaribe and Tamiami viruses with chymotrypsin (Gard et al., 1977) which specifically remove the spikes from the virus surface, as seen by the electron microscope or by a change in the virus density. Polyacrylamide gel electrophoresis (PAGE) analysis of these spikeless virus particles has shown a great reduction of the glycoprotein. A 84,000 m.w. glycoprotein found in preparations of Mopeia 20410 may be a precursor of the GP-1 and/or GP-2 proteins (Kiley 1981). Kiley further suggested that the uncleaved glycoprotein of M20410 may play a role in the differential pathogenicity of the Lassa or Mopeia viruses in experimental animals.

4.2. Serological and Immunochemical Properties (Table 57)

Although the nature of arenavirus surface antigens has yet to be defined it has been suggested by Howard and Simpson (1980) that the

TABLE 57. SUMMARY OF THE SEROLOGICAL AND IMMUNOCHEMICAL PROPERTIES COMMON (a) TO LASSA (LGA 391) AND MOPEIA VIRUSES AND (b) TO LCM AND 'NEW WORLD' ARENAVIRUSES

	(a)	(b)
Common antigens		
CF	+	±
IF	+	±
Neutralizing	-	ND
Common reactive structural proteins		
N	+	ND
G1	?	ND
G2	-	ND
Common reactive nucleoprotein determinants with hybridoma antibodies to LGA 391		
35	+	-
41	+	-
47	-	-
52	-	-
65	+	-
72	+	-
79	-	-
Common reactive nucleoprotein determinants as indicated with hybridoma antibodies to M20410		
53-29-1	-	-
52-153-3	-	-
52-54-6	+	-
53-237-5	-	-
Common reactive nucleoprotein determinants as indicated with hybridoma antibodies to Pichinde		
75-7	+	+
75-19	+	+
75-23	+	+
75-25	+	+

ND - not done
+ = present - = absent ? = unknown

surface glycoproteins play an important role in eliciting a protective antibody response. It remains to be established if each protein is present in all the outer projections or whether they are composed of two or more chemically different types. The role of minor structural components in the formation of antigenically reactive surface components is at present unclear. Biological activities such as haemolysis, haemagglutination etc. which are associated with the envelope protein of other RNA viruses, have not been discovered with arenavirus preparations. Relating the structural aspect of the virus to biological function is the next stage in our understanding of arenavirus infections.

Studies of the immunologic properties of arenaviruses and the varied disease states associated with infection must consider the virus as a complex, replicating mosaic of antigens. Viral antigens are found in many forms during the infection: (a) as free virions, (b) as antigens expressed on the surfaces of infected cells, and (c) as products liberated by degradation of virions or by immunologically mediated destruction.

The most extensive studies on the antigenic properties of the arenaviruses have been primarily investigated with LCM virus (Pederson, 1973) and Pichinde (Rawls and Leung, 1979). Early studies with LCM established that there was a complement-fixing (CF) antigen distinct and separable from the infective particle by centrifugation and designated the soluble antigen (Smadel et al., 1942; Lehmann-Grube, 1971, 1975; Rawls and Leung, 1979). Virus specific CF antigen has further been demonstrated in Junin infected animals (Coto, 1974), from LCM virus-infected tissue cells (Bro-Jorgensen, 1971; Geschwender et al., 1976) and Lassa-infected animals and cells (Buckley, 1973).

Studies presented by Geschwender et al. (1976) on LCM virus and Buchmeier et al. (1977) on Pichinde virus demonstrated that the CF

antigens of these viruses are antigenically identical to an internal structural component of the virion. This component is released by solubilization of virions, and in the solubilized form is resistant to heat denaturation and to proteolysis (Buchmeier et al., 1977). Antisera prepared against purified cell-associated CF antigen of Pichinde specifically immunoprecipitates with the viral nucleoprotein. Immunization with purified nucleocapsid protein of LCM produces a high CF antibody titre, but will not neutralize virus and reacts only with antigens expressed in the cytoplasm of infected cells (Buchmeier and Oldstone, 1978). The mechanism by which excess CF (i.e. nucleocapsid) antigen is released from the infected cell to become 'soluble' antigen is unknown. However, there is no reason to believe that the other cell-associated viral antigens should not have CF activity, this being attributed to the sensitivity of the assay. This view is emphasised by the fact that purified LCM virus could not be lysed by antibody and complement (Welsh et al., 1976). The CF antigens from LCM virus infected tissue and tissue culture cells are immunologically identical (Bro-Jorgensen, 1971; Geschwender et al., 1976). Occasionally two viral antigens related to CF antigen have been found by immunodiffusion. One of these antigens is heat stable and pronase resistant, and has a molecular weight of 20,000-30,000 (Buchmeier and Oldstone, 1979); the other is thermolabile, and has a molecular weight of about 48,000 (Bro-Jorgensen, 1971). Since the arenavirus N protein has a molecular weight of about 66,000 the low molecular weight antigens with CF antivity probably represent degraded N protein. Immunofluorescent studies have shown that the CF antigens in LCM virus-infected cells gives rise to a coarse fluorescence in the cytoplasm of infected cells (Rutter and Geschwender, 1973; Geschwender et al., 1976). Pulse-labelling experiments (Buchmeier et al., 1978) showed that N antigen was synthesised in the cells six hours after the infection, corresponding to the

beginning of the log phase of viral replication. It was not evident on the cell surface.

Neutralizing antibody to LCM is almost certainly directed against one or both of the virus surface glycoproteins. Sera containing high neutralizing antibody titres immunoprecipitate GP-1 and GP-2 glycoproteins. Whether these surface antigens are identical to those recognized by cytotoxic T lymphocytes is a subject of ongoing studies. Evidence from studies in which cytotoxic T cell-mediated lysis of infected cells was blocked by antisera containing GP-1 and GP-2 antibodies but not by monospecific anti-nucleoprotein suggesting that the surface glycoproteins are important in T cell cytolysis (Welsh and Oldstone, 1977; Buchmeier et al., 1978). Monoclonal antibodies generated to all the viral polypeptides can be used as molecular probes to examine this and other questions of specific polypeptide participation in LCM induced disease.

Examination of the serological and immunochemical characteristics common to Mopeia and Lassa viruses are examined and the results summarised in Table 57.

The arenaviruses are serologically related among themselves but not to the viruses outside the family; the present study and those directed to other Lassa viruses (Buckley et al., 1970; Wulff et al., 1978), have clearly established this point. The serological relationships among arenaviruses were observed before morphological and biochemical studies were initiated (Mettler, 1963) and resulted in the creation of the Tacaribe antigenic group, to which additional viruses were subsequently added. The degree of cross-reactivity depends upon the serological test employed. The complement fixation tests exhibits the broadest relationships. LCM, Mopeia and Lassa are distantly related in the CF test to Amapari, Junin, Parana, Latino, Machupo and Tacaribe viruses. Pichinde and Tamiami are not so closely related to each other or to other members of the Tacaribe

complex viruses, whereas Lassa virus seems to be distantly related (Casals et al., 1975; Casals, 1975). Mopeia strains studied in this thesis showed a close relationship to each other and to Lassa (LGA 391) and also demonstrated a distant relationship to LCM (Table 57).

The indirect immunofluorescence test shows a closer relationship among the arenaviruses although the assay system is less specific than the neutralization assay. The assay stains cytoplasmic antigens and demonstrates that Mopeia and Lassa virus are clearly related to each other and more distantly related to LCM and to viruses of the Tacaribe complex (Rowe et al., 1970a; Casals, 1975; Wulff, 1978). Staining the surface of infected cells by IF demonstrated no cross-reactivity among members of the Tacaribe complex or between those viruses and LCM (Buchmeier and Oldstone, 1979). A relationship has been shown by this method between Lassa and isolates from Mozambique (Wulff et al., 1977). These workers also established that both Lassa and the Mozambique isolate (Mopeia 20410) cross react only to a low titre with other members of the arenavirus group. In this study all Mopeia strains react in the same way. Surface staining of Mopeia and Lassa infected cells with anti-Mopeia sera demonstrates a limited IF response, indicating a limited reaction to anti-GP-1 and/or GP-2 antibodies in the antisera so far used. Anti Lassa serum demonstrates some surface staining of both Mopeia- and Lassa-infected cells.

In neutralization assays, arenaviruses form distinct serotypes (Casals, 1975). In those plaque-reduction assays using human serum no cross-neutralization has been observed between viruses of the Tacaribe complex which are closely related by CF, an example being Junin and Machupo viruses, which are quite distinct. A similar marked specificity has been demonstrated between LCM and Lassa viruses, both viruses being readily distinguishable from one another by this technique. However, in

neutralizing assays involving guinea pigs immunized with five doses of Tacaribe virus, anti-Junin neutralization antibodies were shown to develop 20 days after Tacaribe infection demonstrating a high degree of cross-reactivity after 120 days (Weissenbacher et al., 1975/6). Further studies with Tacaribe infection of non-human primates (Callithrix jacchas) demonstrated no anti-Junin antibodies after 45 days, but homologous anti-Tacaribe after 21 days. This type of cross-neutralization relationship between non-infectious Tacaribe and infectious Junin can now be extended to non-infectious Mopeia and infectious Lassa, where present studies show a lack of anti-Lassa neutralization antibodies after Mopeia infection in guinea pigs and Rhesus monkeys. Limited studies of the molecular basis for serological cross-reactivity among Tacaribe complex viruses have been carried out by Buchmeier and Oldstone (1978). Antisera directed against Amapari, Tacaribe and Junin viruses specifically immunoprecipitated only the nucleocapsid protein of Pichinde virus, while antisera directed against Pichinde virus immunoprecipitated the two virion glycoproteins in addition to the nucleocapsid protein. Neutralizing antibody to LCM is almost certainly directed against one or both of the virus surface glycoproteins. LCM sera containing high neutralizing antibody titres immunoprecipitates GP-1 and GP-2 glycoproteins. Present studies have indicated that guinea pig and Rhesus monkey sera raised against LGA 391 reacted strongly with N and G2 structural proteins of all the Lassa and Mopeia strains tested by the "Western blot" assay system (Table 57). This suggests that there are antigenic determinants common to LGA 391 on both the internal nucleocapsid proteins and on at least one of the envelope glycoproteins (G2). No conclusion can be drawn concerning the G1 glycoprotein because of its lack of antigenicity in the "Western blot" assay system. Alternatively, the use of Mopeia antisera demonstrated that the N protein of the Mopeia viruses reacted strongly with antibody, but the N protein of

LGA 391 virus reacted extremely weakly. There was no reaction with the glycoproteins of any virus. Both these characteristics have been endorsed with other Lassa strains (Fig. 55). It is clear that the viruses can be differentiated into two groups: those from West Africa which may be considered Lassa virus strains, and those from Mozambique or Zimbabwe which may be considered strains of Mopeia virus. The reason for the lack of G2 reactivity which is characteristic of all the Mopeia antisera tested so far is unclear. Further work is necessary to determine if antibody reactivity in other systems is present or whether the results reflect an absence of specific glycoprotein antibody. The latter proposal is unlikely since neutralizating antibody has been demonstrated.

Monoclonal antibodies have proved to be useful tools in analyzing virus-specific antigens. The present study demonstrates that antibodies raised in vitro against LCM, Mopeia and Pichinde can be used to analyse the cross-reactive make-up of viruses included in the Arenaviridae. This ability to raise useful cross-reactive monoclonal antibodies may make it possible to circumvent the need to grow dangerous pathogens such as Lassa virus, yet still produce useful and precise clinical reagents. Functional activity of the various anti-glycoprotein hybridomas are as yet not known. Conventional human or animal antisera used in CFT or IF have not been able to differentiate between the Mopeia viruses from Mozambique and Zimbabwe, the Lassa related virus from the Central African Republic or the various West African Lassa isolates. Prior to this Thesis, a limited number of monoclonal antibodies produced by Buchmeier et al. (1980a, 1981) indicated differences between the arenavirus isolates from Africa. However, a cautionary note should be sounded in that it is important to generate a diverse panel of hybridoma antibodies, since one can easily be misled by the results of a small and biased panel of such antibodies which are specific for only limited aspects of the total antigenicity of the viral

protein under examination. Therefore, observations made with individual monoclonals (Buchmeier et al., 1980a) should always be evaluated in the context of the overall antigenicity as delineated by conventional antisera. Nevertheless, a clear differentiation between Lassa and Mopeia viruses can be achieved using nucleocapsid protein-specific monoclonal antibodies raised against Lassa (LGA 391) or Mopeia (M20410) the results of which are summarised in Table 57. When the monoclonal antibodies are tested against Mopeia and LGA 391 viruses, some of the antibodies reacted more or less well with either group of viruses. This pattern was observed using IF or "Western blot" assay systems and and is exactly complementary to that seen by conventional anti-Mopeia antiserum. Taking all the serological and immunological results together, these viruses appear to be serologically related by common antigenic determinants located on the N and G2 proteins, but also possess unique determinants which divide them into two groups. This supports the original hypothesis of antigenic variability between Lassa and Mopeia viruses. Studies using Pichinde monoclonal antibodies also indicate common antigenic determinants between Lassa, Mopeia and Pichinde viruses, and support the inclusion of Mopeia virus within the arenavirus family. The division of Lassa and Mopeia into two groups also coincides with their geographical separation and this is further demonstrated by the host susceptibility studies summarised in Table 58.

4.3. Experimental infection (Table 58)

Almost all the arenaviruses cause a fatal illness in suckling mice following intracerebral inoculation. The exceptions are Latino virus, which does not infect mice, Parana, Lassa and Mopeia viruses which cause illness but few deaths and LCM which has no effect on suckling mice, but produces a fatal infection in young adult mice (Casals 1975). Infection of adult mice with Mopeia and Lassa viruses also proves fatal by the i.c. route

TABLE 58. SUMMARY OF HOST SUSCEPTIBILITY FOLLOWING INFECTION WITH LASSA LGA 391 AND MOPEIA VIRUSES

Host responses	LGA391	Mopeia viruses	
Natural host			
M. natalensis	+	+	
Human susceptibility	Incidental	Unknown	
Acute haemorrhagic disease	+	"	
Pyrexia	+	"	
Lethality %	30-60	"	
Altered serum biochemistry	+	"	
Ab. response	+	"	
Experimental (in vivo)			
Monkey susceptibility	+	-	
Acute haemorrhagic disease	+	-	
Pyrexia	+	-	
Lethality %	100	0	
Altered serum biochemistry	+	-	
Ab. response	+	+	M152*
Guinea pig susceptibility	+	-	(+)
Acute haemorrhagic disease	+	-	(+)
Pyrexia	+	-	(+)
Lethality %	60-70	0	(30)
Altered serum biochemistry	+	-	(+)
Ab. response	+	+	
Adult mouse susceptibility (IC)	+	+	
Acute haemorrhagic disease	+	+	
Lethality %	100	100	
Altered serum biochemistry	+	+	
Ab. response	+	+	
Adult mouse susceptibility (IP)	-	-	
Acute haemorrhagic disease	-	-	
Lethality %	0	0	
Altered serum biochemistry	+	+	
Ab. response	+	+	
Suckling mouse (2-4 day) IC	-	+** -	
Suckling mouse (2-4 day) IP	+** -	-	
Tissue tropism	Pantropic	Occasionally detected in spleen, liver	
Experimental (in vitro)	Both groups of viruses infect a variety of mammalian cell lines		
Site of maturation	Cytoplasm	Cytoplasm	
Mechanism of maturation	Budding through plasma membrane	Budding through plasma membrane	
Cytopathology	Total cell destruction	Partial cell destruction	

* M152 did demonstrate mild Lassa-like characteristics in Dunkin-Hartley guinea pigs.

** Mice became sick with paralysis or stunted growth sometimes leading to death.

+ = present - = absent + = equivocal

but not the i.p. route. The histopathological response observed from i.c. route of infection demonstrated widespread brain lesions but other organs appeared unaffected despite the recovery of high levels of virus. Junin, Latino, Machupo, Parana and Pichinde viruses will kill new born hamsters (Casals, 1975) while guinea pigs are susceptible to Junin (Weissenbacher et al., 1975), Lassa infection (Jahrling et al., 1982) and Mopeia 152 in the present study. Finally Junin (Weissenbacher et al., 1982), and Lassa (Callis 1982; Kiley 1982) have demonstrated the susceptibility of non-human primates. Studies using marmosets challenged with Tacaribe virus (Weissenbacher et al., 1982) and Rhesus monkeys infected with Mopeia strains have shown the non-human primate to be resistant to infection.

The studies reported here suggest that mice, guinea pigs and Rhesus monkeys are potentially useful models for identifying differential aspects of the pathogenesis between Lassa and Mopeia virus infections. They would allow the development of studies into prophylaxis and protection, providing a comparative understanding of the disease process in each model (especially with man).

The disease produced in man, after infection with Lassa, Junin or Machupo follow a similar pattern. The onset of illness is insidious, with chills, malaise, headache, nausea, pain behind the eyes and in muscles followed by fever, conjunctival injection, exanthema and oedema of the face and neck and upper thorax. After a few days the patient becomes appreciably worse with the development of hypotension, oliguria, haemorrhages from gums and nose, haematemesis, haematuria and melaena. Death may result from anaemia, coma, or hypovolaemic shock caused by plasma leakage. In Lassa infections, a pronounced pharyngitis with ulcerative lesions on the tonsils is a frequent finding (P.A. Webb, personal communication). Junin or Machupo are rarely transmitted from person to person like Lassa which has the reputation for frequent man-to-

man transmission. This may correlate with the low titre of circulating virus in patients with AHF and BHF in contrast to the high titre found in some cases of Lassa infection (Emond et al. 1982). Inapparent and sub-clinical infections with Lassa are now believed to be quite common but inapparent infections with Junin and Machupo viruses are extremely rare. J.B. McCormick and P.A. Webb and others (unpublished information) assessed the factors influencing the prognosis of Lassa fever and found that 17 patients died out of 18 patients with serum aspartate transaminase activity in excess of 150 IU/l and a viraemia greater than 10^4 median tissue culture infectious dose/ml. In my investigations, the patient (P.B.) studied in comparison with experimental monkeys survived a transaminase activity of 4440 IU/l and a viraemia greater than 10^5 median tissue culture infectious dose/ml. During the acute stages of Lassa illness, the presence of virus has to date severely restricted haematological and biochemical investigations to guide management. The kidneys of the few Lassa patients studied are variably affected and they show an initial rise in blood urea concentration followed by prolonged haematuria and proteinuria. The lack of evidence of disseminated intravascular coagulation (Copper et al., 1982) suggests that the disturbance of clotting and tendency to bleed were probably related to impaired liver function and vascular endothelial damage, in particular grossly raised serum aspartate transaminase activity and hydroxybutyrate concentrations. In view of lower serum GPT activity, that cannot be the whole explanation. It is more likely that a variety of tissues such as the kidney, muscle and blood also suffer cellular damage and contribute to the high enzyme activities. The prolonged recovery period of the patient under study can be monitored by the enzyme chemistry.

The classical histopathology studies involving Lassa fever, although small in number represent a select and well documented group. A review

(Winn et al.., 1975) demonstrates a major site of tissue damage in the liver and outlines several other areas that deserve further study. There are also interesting parallels with susceptible non-human primates (Rhesus monkeys, Jahrling et al., 1980 and squirrel monkeys, Walker et al., 1982).

The findings of the present study are in general agreement with the results reported by Jahrling et al. (1980) on experimental infection of Rhesus monkeys with the Josiah strain of Lassa virus, the difference being that the Rhesus monkeys studied by Jahrling recovered, a situation not repeated in the present work with LGA 391 infection. There was widespread infection which involved nearly all viscera and caused a prolonged viraemia. The panorganotropism is also comparable to the results of studies of Lassa fever in humans in which adrenotropism, hepatotropism and lymphoreticulotropism have been found (Walker et al., 1982). Moreover as in the disease of humans the development of serum antibody in Rhesus monkeys does not result in rapid clearing of viraemia or assume imminent clinical recovery. The studies of the Rhesus monkey with unmodified Lassa virus infection suggested that damage to certain tissues was variable and that cellular necrosis, host reaction and regeneration occurred in temporal succession. The present studies on clinical biochemistry reflect the degree of pantropic activity with rising levels of enzymes suggesting renal, liver and muscular damage. It is noted by Walker et al. (1982) that pulmonary vasculitis, systemic arteritis and skeletal myositis are more prominent in monkeys than in humans.

Experimental infection of the Rhesus monkey with any of the Mopeia viruses resulted in no adverse clinical or pathological changes. No Mopeia virus could be recovered from the blood at any time post-infection. Anti-Mopeia neutralizing antibodies were evident at 60 days. Immunofluorescent antibodies appeared 12-14 days post infection. A similar situation was noted with Tacaribe infected marmosets except that

anti-Tacaribe neutralizing antibodies appeared 3 weeks post infection (Weissenbacher, 1982). The significance of these two studies stems from the fact that Tacaribe immunised non-human primates are protected against Junin challenge and Rhesus monkeys immunised with any Mopeia virus proved to be protected against Lassa infection.

A similar situation occurs with guinea pig models. Although outbred guinea pigs develop an illness characterised by respiratory insufficiency the mortality rate varies according to the strain of Lassa used (Jahrling et al., 1982; Walker et al., 1975). Organ infectivity titrations showed high titres of Lassa virus in virtually all the organs tested between days 16 and 24 after inoculation. This pattern of organ infection did not suggest any specific tropism, but resembled the 'pantropism' seen in the squirrel and rhesus monkeys.

There was a marked disparity between lesions found in guinea pigs infected with Lassa virus and those found in humans with Lassa fever or in non-human primates experimentally infected with Lassa virus. This was the case in spite of apparently similar patterns of viral tropisms in guinea pigs and primates. Walker et al. (1975) pointed out that disparity in the pathology was seen in the liver and heart. Lassa virus is particularly hepatotropic; however, whereas man develops hepatocellular necrosis, the monkey shows increased regenerative activity and the guinea pig only foci of calcified hepatocytes. Mopeia infection of guinea pigs demonstrates low levels of viral activity in both serum and organs. The main target appeared to be the mesenteric lymph node especially after i.p. inoculation. However, guinea pigs do survive, despite a mild febrile illness after challenge with most Mopeia viruses with the exception of M152. Infection of guinea pigs with M152 resulted in a moderate degree of clinical symptoms similar to Lassa resulting in a 30% death rate. All the symptoms exhibited by Lassa infection are reproduced with lethal M152

infection. Jahrling et al. (1982) have suggested that Strain 13 guinea pigs were more sensitive and were uniformly killed by Lassa, whereas the Hartley strain proved to be relatively resistant. Because of this they suggested that Strain 13 guinea pigs are preferable to outbred animals for studying the pathogenesis of acutely lethal Lassa virus infections and for assessing vaccine and treatment regimes. However, outbred guinea pigs offer an opportunity to generate convalescent antisera and examine the natural basis for fatal versus non-fatal disease, and to study late sequelae (if any) of these infections. These observations are similar to those reported for Pichinde virus which, like Lassa, uniformly killed inbred Strain 13 guinea pigs but killed less than half of outbred animals regardless of the dose (Jahrling et al., 1981).

Lassa virus infections of outbred guinea pigs results in neutralizing antibodies not being detected until eight weeks after the viraemia had subsided. Although antigen concentrations in tissue are high and could conceivably remove neutralizing antibody from the circulation, passively administered antibody was not cleared from the circulation of infected guinea pigs any more quickly than it was from uninfected controls (Jahrling, personal communication). Mopeia neutralizing antibody appeared between 21 and 35 days but no anti-Lassa activity was evident up to 120 days post infection. Differences in susceptibility of guinea pigs, mice and monkeys to Mopeia or Lassa virus are probably also unrelated to the early IFA response, since they were similar in both timing and magnitude. Furthermore early Lassa convalescent sera containing high concentrations of antibody as measured by IFA conferred no demonstrable protection to Lassa infected guinea pigs (Jahrling, personal communication) suggesting that the IFA response is of little consequence in resolving acute Lassa virus infections.

The replication of Lassa virus in tissues showing little or no histological damage or inflammation is a recurrent theme (Jahrling et al.,

1980; Jahrling et al., 1982; Walker et al., 1975). Disease may result as a consequence of functional impairment of infected cells, which appear histologically normal but are inhibited from performing the specialty functions of differentiated cells (Oldstone et al., 1977). Insight critical to the understanding of Lassa and Mopeia virus infections may be obtained by studying the biochemical and haematological changes in blood, hepatic and cardiovascular functions and capillary permeability changes in various animal models. To this end serum biochemistry in all models was studied for both Lassa and Mopeia virus and used as markers in following the progress of viral disease and determining the effectiveness of protection experiments. Numerous experiments with animals including those reported here, together with clinical observations, have demonstrated that the levels of enzyme activity reached in plasma are directly related to the amount of tissue damage, but not necessarily to the severity of the clinical condition. Although little is known at present about the means by which enzymes are cleared from the plasma, enzymes differ in their plasma half-lives and these differences contribute to the observed variations in rates of rise and fall of individual enzymes. It is generally agreed that leakage of enzymes from dying cells is the main cause of the observed rise in serum enzyme activity in such conditions as myocardial infarction and viral hepatitis. Alternate views also suggest that failure to maintain the semi-permeable cell membrane in its normal state is also a cause of enzyme leakage in conditions that stop short of cell death (Kachmer and Ross, 1976 review). The serum enzymes chosen in this study are those considered to be of clinical and diagnostic value. In all experimental models used there is a consistent response to infection in that the enzymes normally associated with cytoplasm (GPT, GOT, LDH) leak into the plasma, progressively increasing until the animal dies or recovers. The enzymes also indicate the target organs affected in that the GOT and GPT are

predominantly an indication of liver damage; LDH and αHBDH activity would indicate the degree of possible damage to liver, heart, kidney, skeletal muscle; γGT activity indicates cardiovascular problems and pancreas malfunction; the CK indicates the degree of muscle damage and normally can be associated with various forms of shock. Therefore the serum enzymes studied verify the 'pantropism' indicated by histopathology and virology, in that Lassa infection as measured by serum biochemical analysis reflects the progress and severity of the disease and are useful markers of the disease process, comparative pathogenesis and assessing the protective ability of a possible vaccine. The degree of damage caused by the Mopeia viruses in the mouse model demonstrated an alteration in serum enzyme activity. This reflected the outcome of the disease and was similar to that found with a Lassa infection. In the case of guinea pigs the levels of serum enzymes remain unaffected upon challenge of these models with all the non-lethal strains of Mopeia virus. The only Mopeia strain not to conform to this non-activity was M152 which caused an increase in GPT, GOT, LDH, γHBDH, AP and CK levels whether there was a lethal or non-lethal outcome, a pattern very similar to Lassa. No Rhesus monkey infected with either a high (6 $\log_{10}TCID_{50}$) or low (2 $\log_{10}TCID_{50}$) dose of any strain of Mopeia virus showed any significant alteration in their serum biochemistry.

The Rhesus monkey known to be highly susceptible to Lassa virus (Jahrling et al., 1981; Walker, 1975; and Walker, 1981) failed to exhibit any clinical symptoms, viraemia or serum biochemical alterations when infected with 10^2 - 10^5 $TCID_{50}$ of Mopeia virus derived from Mozambique or Zimbabwe. A similar situation was also demonstrated with Dunkin-Hartley guinea pigs. It was possible to demonstrate that Mopeia immunised Rhesus monkeys or guinea pigs were fully protected against lethal doses of a pathogenic strain of Lassa LGA 391. Furthermore, in the protected

Rhesus monkey and guinea pig, viraemia was absent together with an inhibition of spread and replication of the challenge virus . This situation is reminiscent of that reported with Tacaribe and Junin, where Tacaribe immunised guinea pigs (Coto et al., 1980; Weissenbacher et al., 1975/76) and marmosets (Weissenbacher et al., 1982) were protected against lethal challenge with Junin. However the systems differ in the humoral antibody studies. It is evident that Tacaribe infected marmosets or Mopeia infected animals were protected by mechanisms that inhibited detectable viral replication, viraemia and spread of challenge virus. Passive immunization studies performed in guinea pigs and humans infected with Junin virus have demonstrated the important role of neutralizing antibodies in the protection against the disease and death induced by Junin (Maiztegui et al., 1979; Weissenbacher et al., 1968). However similar studies carried out in Sierra Leone (P.A. Webb, personal communication) have not proved the efficacy of anti-Lassa convalescent serum in the treatment of Lassa patients. However since neutralizing antibodies develop late in infection the quality of serum used in serotherapy is open to doubt since a true assessment of neutralization antibody content was suspect. P. Jahrling (personal communication) has indicated in guinea pig passive antibody studies that for any serum (human, monkey or guinea pig) to be of any effect the neutralizing index must be at least 2.

The absence of anti-Lassa neutralizing antibodies in Mopeia infected monkeys and guinea pigs up to the time of challenge contrasts with the results obtained in guinea pigs infected with Tacaribe virus, where late anti-Junin antibodies (45 days p.i.) were found. However, in both systems with either guinea pigs or Rhesus monkeys, the protective effect against Lassa was demonstrated before the detection of any humoral neutralizing antibodies. A similar situation was recorded by Weissenbacher et al. (1982) with the Tacaribe-Junin system. It has been generally accepted

that members of the Tacaribe complex are separated by nearly complete absence of antigenic overlap with respect to antigens which determine neutralization (Jahrling et al., 1973). However some findings by Anderson and Downs (1965) and Mackenzie (1965) demonstrated that Junin virus is weakly neutralized by Tacaribe immune serum and that this is a one-way cross-reaction. Weissenbacher et al. (1968 and 1982) demonstrated a two-way cross-reaction using guinea pig and marmoset immune serum. The present study indicates that Mopeia immune sera derived from Rhesus monkeys or guinea pigs did not neutralize Lassa while anti-Lassa serum weakly neutralized Mopeia viruses. Upon challenge of Mopeia immunised animals anti-Lassa neutralizing activity was eventually demonstrated although the level did vary from animal to animal, and was not as dramatic as was found with Tacaribe immune marmosets. Junin challenged anti-Tacaribe animal sera demonstrated levels of LNI >2.

It has been postulated that viral interference or cell mediated immunity (or both) play a part in the protection against both Junin and Lassa in guinea pigs or non-human primates (Coto et al., 1980; Damonte et al., 1978). It is also suggested that cell mediated immunity (CMI) plays a major role in heterologous protection among Togaviruses (Crissman and Hammon, 1974; Jacoby et al., 1980; Latif et al., 1979). This cross-protection occurs in the absenced of detectable cross-neutralizing antibody (Brown and Officer, 1975; Casals, 1965; Chamberlain, 1968; Hearne and Rainey, 1963; Porterfield, 1962; Smith-Owirodu et al., 1980). The suppression of a humoral response to the challenge of Lassa in non-human primates immunised with Mopeia viruses is a significant finding but not unique within the arenavirus group. Weissenbacher et al., (1975/6) reported that infection with viruses of the Tacaribe complex bestowed partial immunity to guinea pigs and modified the course of infection with Junin virus, i.e. 30% of Machupo immunised guinea pigs survived; 3 doses of

Ampari protected 58%. In the present study, the delayed response in neutralizing antibody production to Lassa is coincident with protection similar to that reported between Semliki Forest virus and Sindbis (Smith-Owirodu et al., 1980; Peck et al., 1975). In the Togavirus studies, T-cell mediated immunity plays a major role in cross-protection since recipient animals receiving adoptive T-cell rich populations of spleen cells from an immunized donor resisted fatal challenge (Peck et al. 1979a and b).

Pathologic lesions of human and non-human primates infected with other arenaviruses (Walker et al., 1975) suggest a possible role for immunopathologic mechanisms for the vascular lesions in these Rhesus monkeys. The lymphoreticulotropism of Lassa virus as indicated by lymph node and spleen infectivity titres and necrosis of B-cell regions, may correlate for the delayed neutralizing antibody response which is characteristic of several arenavirus infections of man and experimental animals including those of this present study (Carey et al., 1972; Fraser et al., 1974; Hotchin et al., 1969; Johnson 1973; Jahrling et al., 1982; Weissenbacher 1980). Complement fixing antibody develops slowly in human Lassa fever patients and in the present study none was found, although the monkey did die early. However, Jahrling et al., (1980) and Walker (1975) did not find any neutralizing antibodies 28 days post infection. It has been suggested by Walker et al., (1975) that the delay in anti-Lassa antibody responses could be due to viral infection and destruction of responding lymphoid cells. Another feature of non-human primate lymphoreticulum histopathology was hyperplasia of the T-cell-dependent paracortical regions of the lymph nodes and varying degrees of severity in sinus histocytosis. The precise role of cell-mediated immunopathology in man and hon-human primates is not known. Laboratory models of rodent infections with LCM (Nathanson, 1975) and Tacaribe viruses (Doherty and Zinkernagel, 1974) have demonstrated T-cell mediated immunopathology.

Thus the role of the cell mediated immune system needs to be studied more extensively not only in the area of protection but in studying the differences between fatal and non-fatal arenavirus infections. Studies in determining the differences in cellular immune responses, including quantitative differences in reticuloendothelial cell function may explain variations in susceptibility. The availability of models using inbred animals facilitates adoptive cell transfer studies to approach these CMI problems. The susceptibility of various animal models may also depend on genetically determined differences in cellular susceptibility to produce viral infection or defective interfering particles.

4.4. Conclusion

These studies have shown that Mopeia viruses have physicochemical, morphological and structural characteristics similar to the Lassa virus and those viruses characterised within the arenaviridae. The serological and immunochemical data indicate the presence of both common and unique antigenic determinants on Mopeia and Lassa viruses, suggesting two possible groups within the 'Old World' arenavirus classification. This aspect is endorsed by the geographical separation of these viruses and by the differences in host susceptibility, showing Lassa to be pathogenic and Mopeia non-pathogenic. It is therefore concluded that the Mopeia viruses can be incorporated within the arenavirus family and are minor antigenic variants of Lassa.

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