A LABORATORY TECHNIQUE FOR STUDYING THE INSECT TRANSMISSION OF ANIMAL VIRUSES, EMPLOYING A BAT-WING MEMBRANE, DEMONSTRATED WITH TWO AFRICAN VIRUSES

By R. W. ROSS*

From the Virus Research Institute, Entebbe

(With Plate 4 and 2 Figures in the Text)

CONTENTS

I. INTRODUCTION

To show that blood-sucking insects can transmit viruses which cause human diseases, human beings themselves must be used for experiments, or, as this is rarely possible, their place must be taken by suitable laboratory animals. For this purpose an animal species must be available with the following attributes: it must regularly circulate much virus in its blood after infection, and it must be susceptible to infection by the intracutaneous inoculation of a small dose of virus. None of the animal species available possess these attributes during experimentation with many human viruses. Insect transmission can often then be demonstrated by using the indirect method described in this paper.

This technique enables a mosquito to ingest some of the highly concentrated virus present in a suspension of infected mouse-brain and afterwards to emit virus through a bat-wing into fluid which is then inoculated intracerebrally into mice. In the course of experiments with yellow fever virus the author designed and had constructed an apparatus for warming small quantities of blood, retained in tubes by pieces of bat-wing membrane, so that mosquitoes could feed on it. This was

* Now of the Department of Pathology, University of Cambridge.

https://doi.org/10.1017/S0022172400044454 Published online by Cambridge University Press

done primarily to measure the amount of virus emitted by a previously infected mosquito but it also enabled mosquitoes to engorge in the same way on infectious suspensions of mouse brain, and to transmit by biting through membranes into normal blood.

This report describes the application of the technique to demonstrate the laboratory transmission of two viruses which are under study here. The two viruses were isolated during the course of investigations into an epidemic, which was believed to be mosquito borne. An epidemiological account is given by Lumsden (1955) and a few details of the behaviour of the two viruses along with an outline of the work reported here has already been published (Ross, 1953). In this paper, the term Chikungunya virus is limited to that virus lethal only to baby mice and previously called Chikungunya 'B'. The name 'Makonde' is given to the virus lethal to adult mice and aetiologically unconnected with the epidemic (Ross, 1953).

II. MATERIALS AND METHODS

(*a*) *Mosquitoes*

In March 1953, a great number of *Aedes aegypti* eggs were collected in Newala and used by Dr Gillett to found a mosquito colony of this strain in Entebbe. Adult mosquitoes were reared for these experiments from dried eggs derived from the new colony. The females were placed in individual vials (Ross & Gillett, 1950) the morning after emergence, and were used during the next few days. After an insect had engorged fully on an infected suspension (see below) the vial was numbered and stored at 26° C. and 80 % relative humidity until required. It was found that mosquitoes fed more readily at room temperature (about 22° C.) than at 26° C. All insects were inspected twice daily, and when they died, lost limbs, or became incoordinated (after anything up to 30 days) they were tested for virus content as follows. To immobilize living active insects and to preserve the virus in dead insects, vials were refrigerated with the mosquitoes inside for periods of less than 48 hrs. Mosquito bodies were then ground in a mortar and suspended in 1 ml. of 10 % serum in saline. The extracts were centrifuged for 20 min. at 3000 r.p.m. and the supernate was inoculated intracerebrally into mice to test for the presence of virus.

(*b*) *Virus strains*

A strain of Chikungunya virus was used for these experiments after five passages in baby mice; it was derived from wild *A. aegypti*.

Two strains of Makonde virus were used, first a strain which had passed through forty-three passages in baby mice and originated from wild *Culex* mosquitoes. A second strain isolated in baby mice and stored as infected brain tissue was used in a further experiment.

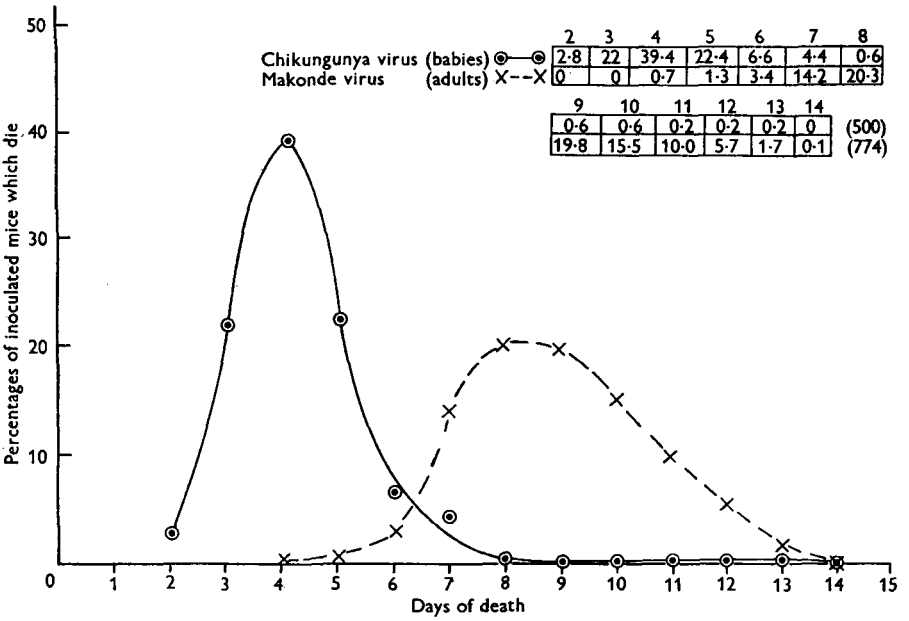
(*c*) *Virus suspensions for infecting mosquitoes*

Six-day-old mice were inoculated intracerebrally with a 1/10 dilution of each strain of virus to provide the infected brain material on which mosquitoes were to be fed. Mice seen to be sick were killed and their brains removed and suspended in 30 % normal rabbit serum in saline.

(*d*) *Titration and detection of virus*

The virus suspensions used for feeding mosquitoes were titrated at the beginning and end of feeding. In experiments with Chikungunya virus, litters of five 6-day-old mice were used for each dilution or each mosquito suspension; animals receiving an inoculum of 0·02 ml. intracerebrally. The mice were examined daily for fourteen days and the survivors counted. Mice eaten by their mothers 48 hr. or more after inoculation were regarded as suffering from virus encephalitis, and were counted as deaths in the calculation of results. Mice dead or eaten earlier were excluded from the calculation.

For experiments with Makonde virus, groups of six 28-day-old mice were used, each animal receiving an inoculum of 0·03 ml. intracerebrally. These mice were kept for 16 days and the survivors were counted; this period was chosen because



Text-fig. 1. The percentages of mice inoculated with two viruses which die on a particular day.

examination of the records of titrations involving the deaths of over 700 mice from Makonde encephalitis showed that 99·8 % of deaths from virus infection occurred before the sixteenth day (Text-fig. 1).

(*e*) *Apparatus*

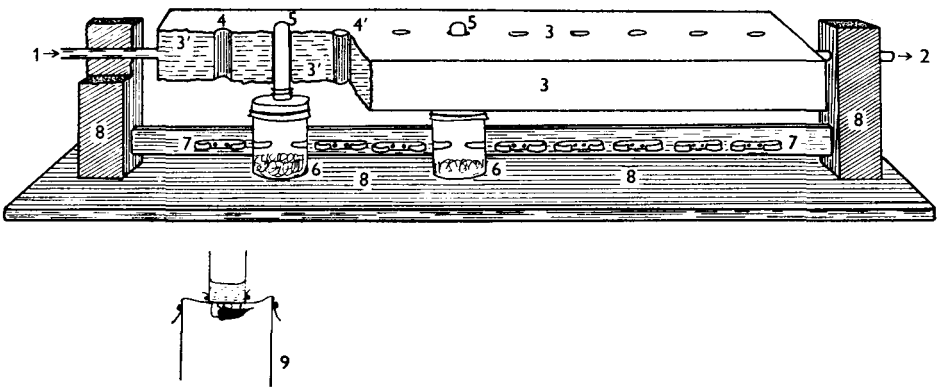
The apparatus used in these experiments has already been described (Ross, 1953). It served to warm small volumes of blood enclosed in tubes by pieces of bat-wing membrane in a position where mosquitoes could engorge upon them (Pl. 4).

It consisted of a rectangular water jacket pierced by ten brass tubes fitted closely round inverted $2\frac{1}{2} \times \frac{5}{8}$ in. Pyrex glass tubes, hereafter referred to as sample

https://doi.org/10.1017/S0022172400044454 Published online by Cambridge University Press

tubes. Water from a 40° C. bath was circulated through this jacket by a pump. The jacket was mounted on a wooden stand fitted with spring clips, which held the special vials in which the mosquitoes were stored in apposition to the ends of the sample tubes (Text-fig. 2). The apparatus was warmed by running the pump for about 15 min. before use. The sample tubes were warmed in a rack in the water bath, and the membrane was applied just before they were put into their places.

To supply the membranes wild bats of several species were collected in the Institute attics shortly before use. A piece of the wing membrane from a freshly killed bat, taken near the free border adjoining the flank, was stretched over the mouth of a sample tube and held in place by several turns of a rubber band. The security of the seal was ensured by leaving a rim of fur and a piece of meta-carpal bone, and the redundant portion of wing was snipped off. Ten previous attempts



Text-fig. 2. Diagrammatic view of apparatus. (1) Warm water intake pipe. (2) Exit pipe. (3) Copper jacket shown in optical section at 3′. (4) Brass pipe shown in section and complete at 4′. (5) Sample tube in position. (6) Mosquito vial in position. (7) Clips for holding mosquito vials. (8) Wooden base plate and uprights of stand. (9) Diagrammatic view of mosquito feeding through a membrane.

to isolate virus from bats-wing membranes by grinding up pieces and inoculating mice with them, yielded no lethal agent. Moreover two large unsuccessful transmission experiments were done, one with neurotropic yellow fever virus and one with Makonde virus, in which 350 samples of normal serum were in contact with pieces of bat-wing through which mosquitoes had probed, and in not one was any kind of virus recovered by mouse inoculation.

(*f*) *The infection of mosquitoes*

The suspensions of infected mouse-brain were used either immediately after harvesting or, in the first experiment with Makonde virus, after storage at −20° C. for up to 48 hr. To a 10 ml. volume of brain suspension, 0·2 ml. of fresh packed rabbit blood cells was added. When these cells were present mosquitoes fed more readily, could then be identified more easily, and digested this concentration more rapidly than whole blood. 0·5 ml. aliquots of the warmed infectious suspension were transferred to sample tubes which were sealed with bat-wing and inverted into the holes in the water jacket, where each was supported by the gauze-covered

upper end of a vial containing a mosquito, which was thus enabled to feed. As soon as a mosquito appeared fully engorged (usually a rapid process), the vial was removed and replaced by a fresh one. Those mosquitoes which did not feed well were discarded. The vials of distended insects were numbered and returned to controlled conditions.

(*g*) *The demonstration of transmission*

After appropriate incubation, mosquitoes from the numbered vials were applied as before to membrane-sealed sample tubes containing 0·5 ml. aliquots of warmed normal serum and 1% blood cells. When an insect had finished probing or engorging the vial was removed. The sample tube was then righted and the contents carefully mixed, the membrane was removed and a cotton plug inserted instead. 0·05 ml. of fluid removed from this tube was mixed with 0·45 ml. of 10% normal rabbit serum for later titration. Mice were inoculated with undiluted fluid; the remainder and the dilution for titrations were stored at $-20°$ C.

(*h*) *Serological confirmation of the presence of virus*

Confirmation of the presence of a particular virus in the brain of an infected mouse was given both by the infection of another group of mice by subinoculation with a Seitz filtrate of brain material in high dilution, and by the survival of a further group after receiving a more concentrated inoculum mixed with specific antiserum. Those fluids which contained enough virus to kill all mice inoculated were titrated to determine the quantity of virus transmitted. A number of preliminary experiments showed that small amounts of virus introduced into similar sample tubes showed no decline of infectivity.

III. RESULTS

(*a*) *Transmission of Chikungunya virus*

Thirty mosquitoes were used in an experiment with Chikungunya virus. They fed on a suspension of mouse brain with an infectivity titre of $10^{-7·2}$ and were then stored under controlled conditions until their deaths. Transmission attempts were made at room temperature on the 14th, 19th, 20th, 21st, 22nd, 23rd, 26th and 28th days following infection. Each mosquito was tested individually for the presence of virus. One mosquito retained no virus and three others contained insufficient to kill all the mice inoculated with extracts of their bodies, but the remaining twenty-six contained enough to kill all mice inoculated, showing that their virus content was more than 50 LD_{50}. (Each mosquito was extracted in 1·0 ml., the mice inoculated with this each receiving 0·02 ml. intracerebrally). The last surviving mosquito retained virus up to 30 days following infection.

In transmission attempts twenty-four infected mosquitoes bit through membranes fifty-one times, and six of these mosquitoes were able to transmit the virus from one to four times each. Seven mosquito bites each introduced enough virus into a sample to kill all the mice inoculated. The stored remainders of these samples were later titrated. No virus was recovered from the diluted samples of

two of them but the others contained from 40 to 130 LD_{50}. Titrations were not done on the other seven samples because in each case some of the mice inoculated with them survived, indicating that individual mice received about 1 LD_{50}, and consequently the whole samples each contained about 25 LD_{50}. Altogether transmission was successfully demonstrated four times by mosquito AA 12, three by AA 21, twice each by three mosquitoes and once by AA 29. AA 12 and AA 21, however, failed to transmit on one further occasion each. Eighteen more infected mosquitoes fed a total of thirty-five times without injecting detectable virus.

To recapitulate, 96·7 % of these mosquitoes retained the virus, and six out of twenty-four were able to transmit by bite.

Table 1. *Number of LD_{50}'s introduced by an infected mosquito*

Mosquito no.	Incubation periods of mosquitoes (days)	Number of LD_{50} from each mosquito
AA 21	23	25*
AA 21	14	25*
AA 12	26	40
AA 12	23	40
AA 12	21	50
AA 20	19	110
AA 23	14	130

* Diluted samples inactivated by storage before titration was done.

Table 2. *Proportion of mosquitoes infected in each batch*

Day of feeding	Infectious titres of mouse brain suspensions	Proportion infected	Percentage infected
1	6·8	44/127	35
2	5·7	13/25	52
3	6·5	15/25	60
Total	Mean 6·3	72/177	56·7

(*b*) *Makonde virus*

(i) *The attempted transmission of a mouse-adapted strain.* The first experiment with this virus was done with 177 mosquitoes which were infected in batches on three successive days on a suspension of mouse brain. Each insect was tested individually for the presence of Makonde virus after death or if judged incapable of sustained feeding. Seventy-two out of the 177 retained the virus, some of them for 28 days, making an infection rate of 56·7 %.

The three batches showed a great difference in the proportion infected, and this rose to its highest in those fed on the third day. The mosquitoes used were hatched from a single large batch of eggs and were used in order of hatching. This point may have some bearing on the proportion infected, since the titre of virus which they ingested only differed slightly from day to day (Table 2).

Batches of from one to ten mosquitoes were fed daily from the 1st to the 28th day following infection to find on what day transmission could first occur. The 153 fluids upon which they fed were tested for the presence of virus. After

excluding fluids bitten into by uninfected mosquitoes, sixty-seven remained; of these thirty-seven are of particular importance because the twenty-three mosquitoes which bit into them had previously survived at 26° C. for 14 days or longer. This period should be long enough to allow of multiplication of the virus, but not one of these fluids contained detectable virus.

In addition to the transmission attempts, weekly samples of ten mosquitoes were killed, each mosquito being titrated individually; only thirteen of these mosquitoes were later proved to contain virus. Table 3 gives logarithms of the number of LD_{50} they contained. Although the number of observations is very small, high values are found in the mosquitoes which have had the longest incubation; a finding consistent with the occurrence of multiplication of the virus in the body of the mosquito.

(ii) *The transmission of an unmodified strain.* As the modified strain was not transmitted, a further experiment was done with a strain which had had only a minimum amount of laboratory manipulation. This strain of Makonde virus was

Table 3. *Amount of virus in mosquitoes after incubation for different periods*

Mosquito no.	Incubation period in days	Log of LD_{50}'s in each mosquito	Mosquito no.	Incubation period in days	Log of LD_{50}'s in each mosquito
M 8	7	1·7	M 75	21	4·1
M 14	7	2·5	M 102	28	3·6
M 57	14	1·7	M 113	28	4·1
M 34	14	1·8	M 69	28	5·2
M 68	14	3·5	M 67	28	5·2
M 85	21	2·1			

isolated from a single mosquito allowed to feed on a patient in Newala. It was used after only two passages in mice because work by Davis, Lloyd & Frobisher (1932) showed that the ability of mosquitoes to transfer yellow fever virus by bite diminished as the number of mouse passages increased, and perhaps this effect accounted for the negative results of the previous experiment.

Twenty-nine insects were fed on a suspension of an infectivity titre of $10^{-5\cdot9}$; of these 15 (51·7 %) were shown to retain virus, one of them up to the 37th day. Transmission attempts were made on the 15th, 18th, 20th, 22nd and 25th days after infection and ten of the infected insects were induced to feed a total of twenty-four times. One mosquito bit and failed to transmit on the 18th day but transmitted probably on the 20th and certainly on the 22nd day. The transmission on the 20th is counted as a probable only, because a single mouse died with typical symptoms and serological confirmation was omitted. The transmission on the 22nd day was confirmed by the neutralization of over 1000 LD_{50} of virus in the brain of an infected mouse.

In the first experiment with mouse-adapted Makonde virus 56·7 % of mosquitoes were infected and the variation between batches was from 35 to 60 %. 51·7 % of mosquitoes were infected in the second experiment, a figure well within the previous range.

As regards transmission, twenty-three mosquitoes failed to transmit the mouse-

adapted strain on the thirty-seven attempts which were made after an adequate incubation period, and one mosquito passed the unadapted strain, while nine others failed on twenty-two occasions.

IV. DISCUSSION

In the study of mosquito-borne diseases a number of authors have described techniques for infecting mosquitoes by imbibing fluid soaked up in cotton wool (Merrill & Tenbroek, 1935; Hammon & Reeves, 1943). Such techniques enable a virus from an animal host to infect mosquitoes, even if it is not present in the circulating blood, but is localized in an organ such as the brain. In such methods the exterior of a mosquito body can be contaminated with the virus. Whitman & Antunes (1938) demonstrated the infection of adult mosquitoes with yellow fever virus after they had been immersed for a short time in infectious serum of high titre whilst in the larval stage. Adult mosquitoes are probably as susceptible as larvae to infection by external contamination. This drawback is eliminated by the use of an animal membrane as described by Bishop & Gilchrist (1946) for studies with avian malaria. Even after mosquitoes are infected, however, transmission by bite can be directly demonstrated only if there is a suitable species of laboratory animal susceptible to a small dose of virus by a peripheral route. In the case of Chikungunya and Makonde viruses no species of those available was susceptible in this way (Ross, in preparation).

The membrane technique described here overcame both the difficulty of infecting mosquitoes by bite and that of demonstrating infection in relatively insusceptible animals. Its use enabled groups of animals to be inoculated by their most susceptible route, instead of individuals bitten peripherally, thus standardizing results. Also the measurement of the inoculum emitted by a single mosquito—otherwise difficult and inaccurate—became easy. This measurement is important because only those animal species susceptible to infection by a peripheral route with an inoculum of the right order of size are able to take part in a natural cycle.

Transmission demonstrated by this technique bears little resemblance to transmission in nature, but this reservation also applies, though perhaps in a lesser degree, to all experiments with animal viruses grown in unnatural hosts. Moreover, in the difficult matter of virus concentration while the mosquitoes are being infected, the membrane technique has a marked advantage over the usual method. The duration and intensity of viraemia in most virus diseases is short and variable and may finish on the appearance of antibody and before the death of the host. The period is consequently difficult to predetermine. In the brain of an infected mouse virus multiplication occurs late in the disease and reaches a plateau rather than a peak, which may persist until the death of the animal. Baby animals were used to provide infective suspensions because in them a high titre of virus was regularly produced in a time too short to allow of the appearance of antibody. That the titres used were of the same order of magnitude as those found in human sera was suggested because the one titration done on pooled lyophilized and rehydrated serum had a titre of 10^{-7} and contained both viruses (Ross, in preparation).

REFERENCES

BISHOP, A. & GILCHRIST, B. M. (1946). Experiments upon the feeding of *Aedes aegypti* through animal membranes with a view to applying this method to the chemotherapy of malaria. *Parasitology*, **37**, 85.

DAVIS, N. C., LLOYD, W. & FROBISHER, M. N. (1932). Transmission of neurotropic yellow-fever by *Stegomyia* mosquitoes. *J. exp. Med.* **56**, 853.

HAMMON, W. M. & REEVES, W. C. (1943). Laboratory transmission of St Louis encephalitis virus by three genera of mosquitoes. *J. exp. Med.* **78**, 241.

LUMSDEN, W. H. R. (1955). An epidemic of virus disease in southern province, Tanganyika territory, in 1952–53. *Trans. R. Soc. trop. Med. Hyg.* **49**, 33–57.

MERRILL, M. H. & TENBROEK, C. (1935). The transmission of equine encephalomyelitis by *Aedes aegypti*. *J. exp. Med.* **62**, 687.

ROSS, R. W. & GILLETT, J. D. (1950). The cyclical transmission of yellow-fever virus through the grivet monkey, *Cercopithecus aethiops centralis* Neumann, and the mosquito *Aedes* (*Stegomyia*) *africanus* Theobald. *Ann. trop. Med. Parasit.* **44**, 351–356.

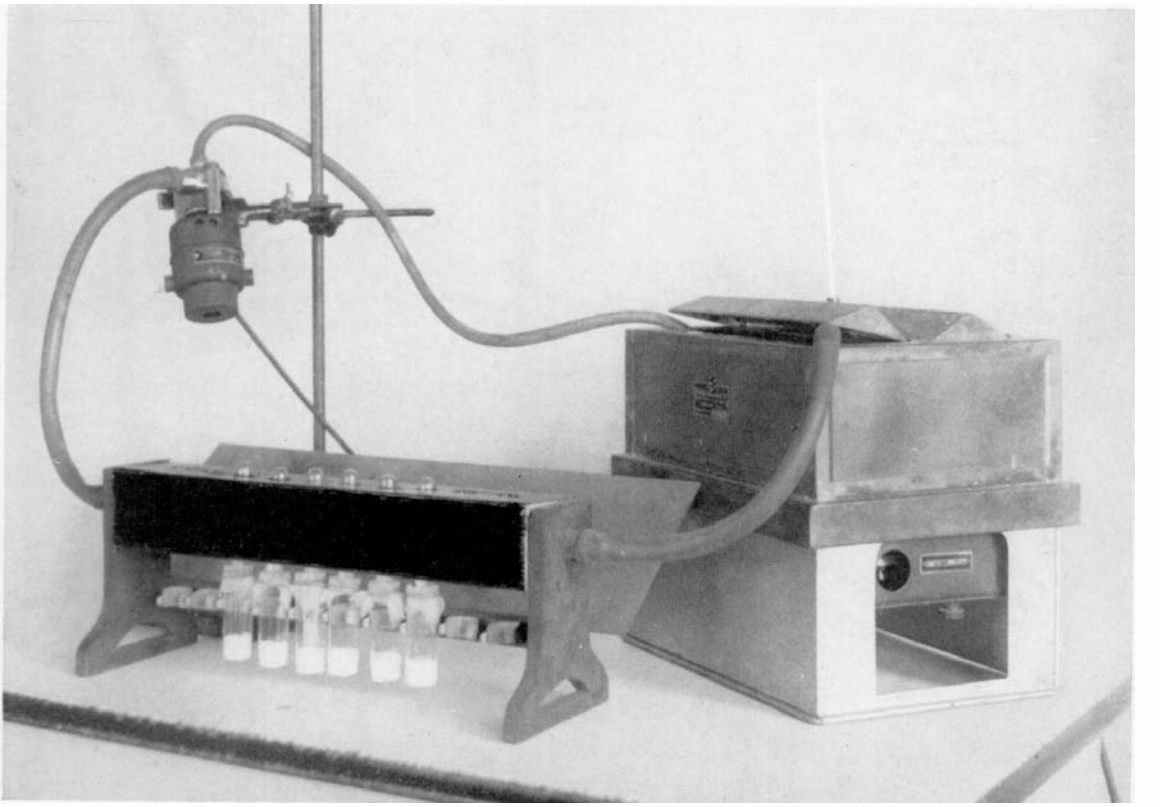
ROSS, R. W. (1953). *Annual Report Virus Research Institute, Entebbe.*

WHITMAN, L. & ANTUNES, P. C. A. (1938). Studies on *Aedes aegypti* infected in larval stage with virus of yellow fever. *Proc. Soc. exp. Biol., N.Y.*, **37**, 664–6.

EXPLANATION OF PLATE

General view of apparatus showing arrangement of pump, water-bath and jacket.

(*MS. received for publication* 17. x. 55)



(*Facing p.* 200)

https://doi.org/10.1017/S0022172400044454 Published online by Cambridge University Press