THE TITRATION OF YELLOW FEVER VIRUS IN STEGOMYIA MOSQUITOES*

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To workers engaged in the study of yellow fever it has always been a question of interest whether the virus multiplies in its insect host. Gay and Sellards (1) in 1927 plotted a conjectural curve showing an increase of virus in mosquitoes for 3 weeks after the ingestion of infectious blood; the initial increase was followed by a constant level. Recently, Davis (2), in discussing the early development of infectivity in stegomyia mosquitoes at temperatures of 30°C. and above, gave as a probable explanation the rapid multiplication of virus at those temperatures. The present series of titration experiments shows conclusively that in the highly efficient insect host, *Aëdes (Stegomyia) aegypti* (Linn.), the quantity of virus present never surpasses that originally ingested.

Methods

It was planned at first to titrate neurotropic yellow fever virus in mosquitoes which had fed on mice or on monkeys at a suitable interval after a massive inoculation with infected mouse brains. Various difficulties supervened. It was found that the neurotropic virus did not reach a high concentration in the blood stream of either species of animal. Feeding of mosquitoes on artificial mixtures of blood and infected mouse brains proved to be impracticable; never did enough insects feed to allow for an adequate series of titration exeriments. For detecting small quantities of virus in mosquito suspensions, the intracerebral route of injection into mice proved superior to the intraperitoneal route; however, irrespective of the route of injection, bacterial contaminants in the lower dilutions frequently

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killed many animals. Filtration introduced an unknown, and probably variable, factor. In spite of the considerable expense involved, it was finally decided to work with viscerotropic yellow fever virus, Asibi strain, and to use *rhesus* monkeys as test animals.

Three series of titration experiments were performed. For the first series about 600 to 700 insects, and for each of the second and third series about 1,000 insects, never previously fed on blood, were allowed to engorge on an infected monkey. For convenience in handling they were distributed among four to six cages. At the beginning of each series all were approximately 2 weeks old. In each initial experiment, before the meal on infectious blood, 120 mosquitoes were weighed alive, 10 at a time, in celluloid catching tubes. In the first and third series honey and water remained in the cages until the hour of feeding; in the second series food and water had been removed about 16 hours beforehand. The infected (febrile) monkey was left in each cage for 1/2 hour. The amount of blood ingested was determined at the end of the feeding period from the increased weight of 100 mosquitoes intended for titration in the first experiment. For this, as well as for subsequent experiments, representatives were caught from all cages. Mosquitoes that had failed to take blood were destroyed. Except for such blood meals as will be mentioned in the protocols, the infected lots of mosquitoes received routinely only honey for food; wet cotton in each cage furnished water.

One hundred infected mosquitoes were titrated in nearly every experiment. For Experiment 5 of the first series only 90 mosquitoes were available; for Experiment 6 of the second series only 87 mosquitoes were still alive. The insects were anesthetized with chloroform at the time of catching; many of them were not actually killed until they were gound in the mortar. Except in the early experiments of the first series they were mashed into a paste with a few drops of undiluted normal monkey serum; to the paste was added 0.5 gm. of finely powdered pyrex glass (this material for grinding was chosen at the suggestion of Dr. J. H. Bauer). The glass had received a preliminary treatment with strong sulfuric acid, had been washed many times with distilled water, and had been dried in a porcelain dish over a flame. In small La Motte cups for testing hydrogen ion concentration, the addition of this powder to distilled water of pH 6.9 changed the reaction to pH 7.1. Grinding of the mosquitoes after the addition of powdered glass occupied about 3 minutes.

The lowest dilution in each case was 1:100 (except in the first series, where an error in calculation was made). Dilution was based on volume of blood ingested, determined from the weight, using the factor 1.06 as the approximate specific gravity of monkey blood. Included in the calculated total of dilution fluid were the few drops of whole serum already added before grinding; the rest of the fluid was 10 per cent normal monkey serum in physiological saline solution. Approximately one-half of this quantity was saved for rinsing the mortar and pestle. The total time required for grinding and diluting was 6 to 7 minutes. Dilutions in all experiments subsequent to the first one in each series were made as if the mosqui-

toes contained at the moment the original amount of blood ingested. That is, in a given series the amount of dilution fluid was the same in every experiment. The 1:100 suspension of ground mosquitoes was lightly centrifuged for 3 minutes. An attempt was made to have the rate of centrifugalization the same each time, but probably this uniformity was only approximate because of variations in strength of electric current. From the somewhat turbid supernatant fluid higher dilutions were made with 10 per cent normal monkey serum, using a clean sterile pipette for each successive dilution.

Monkeys, usually in duplicate, were inoculated subcutaneously with 1 cc. of each dilution. In no instance did an unnecessary delay occur; injections were begun within 1/2 hour of catching the mosquitoes. Temperatures of the animals were taken twice daily during the period of observation. At the end of about 3 weeks survivors were bled for a test of immunity, but were still kept under observation. Prolonged surveillance was necessary, because five monkeys died with typical yellow fever at 3 weeks, or more, after inoculation; the two latest deaths were at 29 and at 27 days, respectively. In all cases of death, confirmation of yellow fever was made by microscopical examination of liver sections. Sera from survivors were tested against neurotropic virus in mice. Animals whose sera gave protection, and those whose sera were inconclusive, were given test doses of potent viscerotropic virus as a further check on the presence of immunity. Those whose sera were definitely negative by the mouse test, and which survived an additional period of observation without fever, were considered suitable for use in later experiments.

Results of Mosquito Titrations in Monkeys

Series I (Begun February 15, 1932).—The average weight of 120 unfed mosquitoes chosen as a sample, 30 from each of four cages, was 2.805 mg. The average weight of blood ingested by 100 engorged mosquitoes used in the first experiment, was 1.25 mg.

Table I summarizes the results of titrations immediately following engorgement, and at intervals of 4, 14, and 28 days thereafter. The immediate end-point (first experiment) was at a dilution of 1:18,000,000. The end-point in the two succeeding experiments (at 4 and at 14 days) was at a dilution of 1:36,000. The end-point in the fourth experiment (at 28 days) was at a dilution of 1:1,800,000.

To account for the rise in titer from the third to the fourth experiment several factors had to be considered, which conceivably may have influenced results: (*a*) the passage of time (a fortnight between experiments); (*b*) a single meal on normal monkey blood 2 days before the fourth titration (3); (*c*) the addition in the fourth experiment of 3 drops

TABLE I

Mosquito Titrations, Series I

Dilution*	Experiment 1. Day of infective blood meal				Experiment 2. 4 days after infective blood meal				Experiment 3. 2 wks. after infective blood meal				Experiment 4. 4 wks. after infective blood meal†			
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Monkey	Fever	Death from yellow fever	Day of death following inoculation
1:90	a	+	+	6	a	+	+	7	a	+	−	8	a	+	+	8
	b	+	+	5	b	+	+	8	b	+	+	8	b	+	+	6
	c	+	+	6	c	−	−	−	c	+	+	7	c	+	+	9
	d	+	+	7	d	+	+	7	d	−	−	8	d	+	+	7
1:180	e	+	−	7	e	−	−	−	e	−	−	−	e	+	+	7
	f	+	+	7	f	−	−	−	f	−	−	−	f	+	+	7
	g	+	+	5	g	−	−	−	g	−	−	−	g	−	−	−
	h	+	+	7	h	−	−	−	h	−	−	−	h	−	−	−
	i	+	+	7	i	−	−	−	i	−	−	−	i	−	−	−
	j	+	+	9	j	−	−	−	j	−	−	−	j	+	+	8
	k	−	−	−	k	−	−	−	k	−	−	−	k	−	−	−
1:1,800	a	+	+	6	a	+	+	8	a	+	−	8				
	b	+	+	5	b	+	+	8	b	+	+	7				
	c	+	+	6	c	−	−	−	c	+	+	8				
	d	+	+	7	d	+	+	7	d	−	−	8				
1:36,000	e	+	−	7	e	−	−	−	e	−	−	−				
	f	+	+	7	f	−	−	−	f	−	−	−				
	g	+	+	5	g	−	−	−	g	−	−	−				
	h	+	+	7	h	−	−	−	h	−	−	−				
	i	+	+	7	i	−	−	−	i	−	−	−				
	j	+	+	9	j	−	−	−	j	−	−	−				
	k	−	−	−	k	−	−	−	k	−	−	−				
1:180,000	a	+	+	6	a	+	+	7	a	+	+	−				
	b	+	+	5	b	+	+	8	b	+	+	8				
	c	+	+	6	c	−	−	−	c	+	+	7				
	d	+	+	7	d	+	+	7	d	−	−	8				
1:450,000	e	+	−	7	e	−	−	−	e	−	−	−				
	f	+	+	7	f	−	−	−	f	−	−	−				
	g	+	+	5	g	−	−	−	g	−	−	−				
	h	+	+	7	h	−	−	−	h	−	−	−				
	i	+	+	7	i	−	−	−	i	−	−	−				
	j	+	+	9	j	−	−	−	j	−	−	−				
	k	−	−	−	k	−	−	−	k	−	−	−				
1:900,000	a	+	+	6	a	+	+	7	a	+	+	−				
	b	+	+	5	b	+	+	8	b	+	+	8				
	c	+	+	6	c	−	−	−	c	+	+	7				
	d	+	+	7	d	+	+	7	d	−	−	8				
1:1,350,000	e	+	−	7	e	−	−	−	e	−	−	−				
	f	+	+	7	f	−	−	−	f	−	−	−				
	g	+	+	5	g	−	−	−	g	−	−	−				
	h	+	+	7	h	−	−	−	h	−	−	−				
	i	+	+	7	i	−	−	−	i	−	−	−				
	j	+	+	9	j	−	−	−	j	−	−	−				
	k	−	−	−	k	−	−	−	k	−	−	−				

Dilution*	Experiment 1. Day of infective blood meal	Experiment 2. 4 days after infective blood meal	Experiment 3. 2 wks. after infective blood meal	Experiment 4. 4 wks. after infective blood meal†
	Subsequent immunity	Subsequent immunity	Subsequent immunity	Subsequent immunity
1:90				
1:180			+	
1:1,800				
1:36,000		−	− +	+ +
1:180,000	+		−	+
1:450,000			−	
1:900,000			−	−
1:1,350,000	−		−	

1:1,800,000	l	+	−	−	−	m	−	−	−	−	o	+	−	−	−	n	+	−	−	+‡
	m	+	+	6		n	−	−	−	−	p	−	−	−	−					
1:18,000,000	n	−	−	−	−	o	−	−	−	−	q	+	−	−	−	o	−	−	−	−
	o	+	+	14		p	−	−	−	−										
1:90,000,000	p	+	−	−	−	q	−	−	−	−	r	+	−	−	−					
	q	−	−	−	−	r	−	−	−	−										
1:180,000,000	r	−	−	−	−	s	−	−	−	−	s	−	−	−	−					
	s	+	−	−	−	t	−	−	−	−										
1:900,000,000	t	+	−	−	−	u	−	−	−	−	t	−	−	−	−					
	u	−	−	−	−															
1:1,800,000,000	v	−	−	§		v	−	−	−	−	u	+	−	−	−					
	w	+	−	−	−															

* An error of calculation in the first experiment brought dilutions to odd fractions. To make results comparable, the same process of dilution was used in all experiments. The two lowest dilutions are given to the nearest 10, and higher dilutions correspondingly in round numbers.

† In this Experiment 3 drops of undiluted normal monkey serum was added to the mosquitoes before grinding with powdered glass.

‡ Monkey died 6 days after the last fever without definite lesions of yellow fever. Serum at that time gave positive protection test in mice.

§ Died of dysentery.

of undiluted normal monkey serum to the mosquitoes before grinding with powdered glass.

A sufficient number of mosquitoes remained alive to test the protective value of whole serum. Therefore, a fifth experiment (not included in Table I) was performed. Ninety surviving mosquitoes were killed and distributed equally between two mortars. To one-half, whole serum was added before grinding; to the other half none was added. Both lots were diluted to 1:180,000 with 10 per cent normal monkey serum; and six monkeys were inoculated, three from each lot. Of the monkeys inoculated from the mosquito lot which had received an addition of whole serum before being ground, all three died with yellow fever. Of those inoculated from the duplicate lot of mosquitoes which had not been ground with serum, only one died, and the other two were not immunized. The result indicated that the addition of whole serum before grinding with powdered glass undoubtedly helped to preserve the virus.

Series II (Begun March 28, 1932).—The average weight of 120 unfed mosquitoes chosen as a sample, 20 from each of six lots, was 2.783 mg. The average weight of blood ingested by 100 engorged mosquitoes used in the first experiment was 1.534 mg. In each experiment 0.3 cc. of undiluted normal monkey serum was added to the mosquitoes before grinding.

Table II summarizes the results of titrations immediately following engorgement, and at intervals of 1, 3, 5, and 11 weeks thereafter. The immediate end-point (first experiment) was at a dilution of 1:1,000,000,000. 1 week later the highest dilution causing fatalities was 1:1,000,000. At 3 weeks the dilution 1:50,000,000 killed a monkey. At 5 weeks the highest infective dilution was also 1:50,000,000 for the mosquitoes which had been offered a blood meal four times in the preceding fortnight; for those which had ingested no blood, it was 1:1,000,000. Before feeding any lots between the experiments at 3 weeks and at 5 weeks, a thorough intermixture of mosquitoes was effected among Batches 1 and 4, 2 and 5, 3 and 6; this should have equalized the lots offered blood (Nos. 1 to 3) and those not offered blood (Nos. 4 to 6). During the succeeding 6 weeks no mosquitoes took blood. The combined survivors of all lots (87 mosquitoes) at 11 weeks after infection gave an end-point at a dilution of 1:1,000,000.

Series III (Begun May 30, 1932).—The average weight of 120 unfed mosquitoes chosen as a sample, 20 from each of six lots, was 2.47 mg. The average weight of blood ingested by 100 engorged mosquitoes used in the first experiment was 1.13 mg. In each experiment 0.35 cc. of undiluted normal monkey serum was added to the mosquitoes before grinding.

Table III summarizes the results of titrations immediately following engorgement, and at intervals of 4 days, and of 6 weeks thereafter. The immediate end-point (first experiment) was at a dilution of 1:1-000,000,000. 4 days later the end-point was at 1:10,000,000. After 6 weeks it had risen to 1:100,000,000, both for the mosquitoes which had been offered six blood meals in the interval between experiments, and for those which had received only honey and water in the meanwhile.

Mosquito Titrations in Mice

Athough, as previously stated, none of the titrations of neurotropic virus was satisfactory, the least unsatisfactory experiment may be cited as confirmatory evidence of a reduction of titratable virus in infected mosquitoes.

On Dec. 3, 1931, there was prepared a 33 1/3 per cent suspension of infected mouse brains in diluting fluid (10 per cent normal monkey serum). 1 cc. of the mixture was injected intraperitoneally into each of twelve mice (Groups R547 and R548). Between 2 and 3 hours later Mosquito Lot 675, which had been without food or water for 24 hours, was allowed to feed. From the weights of 100 engorged mosquitoes the average blood meal was calculated at 2.3 mg. With these insects six dilutions were made, the lowest 1:25, the highest, 1:100,000; and each dilution was injected intracerebrally into a group of six mice. Deaths occurred in every mouse group except the last. From the group inoculated with dilution 1:10,000, a positive subinoculation was obtained. The infectivity of the mice upon which mosquitoes fed was tested immediately after the blood meal by the injection of pooled serum from three of these into twelve other mice, all of which died in a typical manner on the 4th and 5th days thereafter.

On Dec. 17, a fortnight after the infective blood meal, 106 of the mosquito lot were killed for the second experiment. Nine dilutions were made, the lowest 1:25, the highest 1:1,000,000, and each dilution was injected intracerebrally into a group of six mice. Deaths occurred among the mice inoculated with dilutions 1:25, 1:100, and 1:500. Subinoculated mice all died early, indicating bacterial contamination. However, even if virus were present up to dilution 1:500, the titer had dropped to 5 per cent of that in the first experiment.

TABLE II

*Mosquito Titrations, Series II**

Dilution	Experiment 1. Day of infective blood meal				Experiment 2. 1 wk. after infective blood meal					Experiment 3. 3 wks. after infective blood meal					Experiment 4. 5 wks. after infective blood meal†					Experiment 5. 5 wks. after infective blood meal†					Experiment 6. 11 wks. after infective blood meal				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100	a	+	+	5	a	+	−	7	+	a	+	+	7															
1:1,000	b	+	−	7	b	+	+	7		b	−	−	++															
1:20,000	c	+	+	7	c	+	+	7		c	−	+	6															
1:100,000	d	+	+	8	d	+	+	11	+	d	+	+	7		a	+	+	5										
1:250,000	e	+	+	5	e	+	+	6		e	+	+	9		b	+	−	−	+									
1:500,000	f	+	+	5	f	+	+	6		f	+	+	6		c	+	+	5			a	+	−	8				
1:750,000	g	+	+	8	g	−	+	8		g	+	+	8		d	−	+	7			b	+	−	9				
1:1,000,000	h	−	+	5	h	+	+	8		h	+	+	6		e	+	+	8			c	+	−	9				
	i	+	+	6	i	+	−	10	+	i	−	+	6		f	+	+	8			d	+	−	17				
1:10,000,000	j	+	+	6	j	+	+	9		j	+	+	7		g	+	+	8			e	+	−	16				
	k	+	−		k	+	−		−	k	+	+	6		h	+	+	8			f	+	−	12				
					l	+	+	7		l	+	+	7		i	+	+	8			g	+	−	14				
					m	+	+														h	+	+					
					n	+	−		−	m	−	+	7		j	+	+	8			i	+	−		−	l	−	−
					o	+	−		−	n	+	−		−	k	+	+	8			j	−	−		−	m	−	−

1:50,000,000	l	+	+	14		p	+	−	−	−	o	+	−	−	−	l	+	+	12		k	−	−	−	−	n	−	−	−	−
	m	+	+	12		q	+	−	−	−	p	+	+	27		m	+	+	6		l	−	−	−	−	o	−	−	−	−
1:100,000,000	n	−	+	5		r	+	−	−	−	q	−	−	−	−	n	−	−	−	−	m	+	−	−	−	p	−	−	−	−
	o	+	+	6		s	−	−	−	−	r	−	−	−	−	o	−	−	−	−	n	−	−	−	−	q	−	−	−	−
1:250,000,000																p	−	−	−	−										
1:500,000,000	p	−	−	−	−	t	+	−	−	−	s	+	−	−	−	q	−	−	−	−	o	−	−	−	−					
	q	−	−	−	+	u	+	−	−	−						r	−	−	−	−	p	−	−	−	−					
1:1,000,000,000	r	+	+	8		v	+	−	−	−	t	−	−	−	−	s	−	−	−	−	q	−	−	−	−					
	s	−	−	−	−	w	−	−	−	−																				
1:10,000,000,000	t	−	−	−	−	x	−	−	−	−	u	+	−	‡							r	−	−	−	−					
	u	−	−	−	−																										
1:100,000,000,000	v	−	−	−	−																										

* In each experiment 0.3 cc. of undiluted normal monkey serum was added to the mosquitoes before grinding.

† Mosquitoes used in Experiment 4 of this series had been offered a blood meal upon four occasions in the fortnight following Experiment 3. Mosquitoes in Experiment 5 were control mosquitoes which had not taken blood since the original infective feeding. Many non-immunizing fevers, particularly in Experiments 2 and 3, were caused by tuberculosis.

‡ Died of intercurrent disease.

TABLE III

*Mosquito Titrations, Series III**

Dilution	Experiment 1. Day of infective blood meal					Experiment 2. 4 days after infective blood meal					Experiment 3. 6 wks. after infective blood meal†					Experiment 4. 6 wks. after infective blood meal†				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100,000						a	+	+	7		a	+	+	7						
1:250,000						b	+	+	9		b	−	+	9						
1:500,000	a	+	+	8		c	+	+	14		c	−	+	7		a	−	+	7	
						d	+	+	7		d	−	+	7		b	+	+	14‡	
1:750,000	b	+	+	6		e	+	+	13		e	−	+	8		c	+	+	5	
						f	+	+	5		f	−	+	9		d	+	+	8	
1:1,000,000	c	+	+	6		g	+	+	5		g	−	+	6		e	+	+	14	
	d	+	+	9		h	+	+	5		h	−	+	17		f	+	+	11	
1:10,000,000	e	+	+	11		i	+	+	5		i	+	+	18		g	−	+	7	
	f	+	+	9		j	+	+	10		j	−	+	22		h	−	+	9	
	g	+	+	11		k	−	−	−	−						i	+	+	−	+
1:50,000,000	h	+	+	5		l	−	−	−	−	k	−	+	−	−	j	−	+	−	
	i	+	+	7		m	−	−	−	−	l	−	+	−	−	k	+	+	18	
																l	−	+	−	−

1:100,000,000	j	+	+	7		n	−	−	−	−	m	−	−	−	−	m	+	+	21	
	k	+	−	−	+	o	−	−	−	−	n	+	+	29		n	−	−	−	−
1:500,000,000	l	+	+	15		p	−	−	−	−	o	−	−	−	−	o	−	−	−	−
	m	+	+	24		q	−	−	−	−										
1:1,000,000,000	n	+	+	6		r	+	−	−	−	p	−	−	−	−	p	−	−	−	−
	o	−	−	−	−															
1:10,000,000,000	p	−	−	−	−	s	−	−	−	−										
	q	−	−	−	−															
1:100,000,000,000	r	−	−	−	−															

* In each experiment 0.35 cc. of undiluted normal monkey serum was added to the mosquitoes before grinding.

† Mosquitoes used in Experiment 3 had been allowed to feed on monkey blood six times in the interval following Experiment 2. Mosquitoes used in Experiment 4 had taken no blood since their original infective feeding.

‡ Death complicated by peritonitis. Midzonal necrosis and intranuclear inclusions present in liver.

DISCUSSION

In 1930 Bauer and Mahaffy (4) and Aragão and da Costa Lima (5) reported titrations of yellow fever virus in infected mosquitoes. One of the experiments of the latter authors may be cited. Three stegomyia mosquitoes which had taken an infective blood meal 1 month previously were ground thoroughly in a mortar and were suspended in

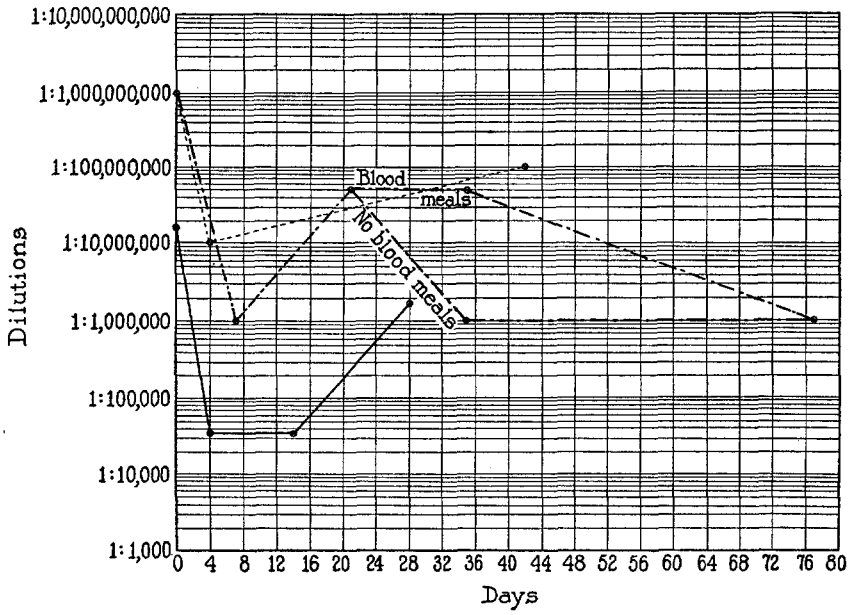

CHART 1. End-points in mosquito titrations.

——— Titrations first series.
—·—·— Titrations second series.
- - - - - - - - Titrations third series.

10 cc. of distilled water. From this first dilution 1 cc. was added to 9 cc. of distilled water; succeeding dilutions were similarly made to a total of six. A monkey became infected following the injection of 1 cc. from the sixth dilution. As the authors remark, if the three mosquitoes had occupied a volume of 1 cc., the final dilution would have been in the neighborhood of 1:1,000,000. However, the mosquitoes probably weighed about 8 mg.; hence the final dilution was approximately

1:125,000,000. Based on the amount of infectious blood originally ingested, the final dilution was probably between 1:150,000,000 and 1:300,000,000. This is a remarkable titer, considering that distilled water was used as the diluting fluid. Nothing is said in the protocol about filtration or centrifugalization to eliminate gross particles from the first dilution (original suspension).

The experiments reported in the present paper probably leave some room for criticism. However, they were as nearly comparable one with another as circumstances permitted. Perhaps the greatest source of error was in centrifugalization of the 1 per cent suspension. In the first experiment of each series all of the virus was contained in freshly ingested blood and was affected scarcely at all by centrifuging. In succeeding experiments it is very probable that some intracellular virus was not released by grinding but was carried down in the debris removed by centrifugalization. This would account in part for the drop in titer during the first week or two after an infective blood meal. A good part of the drop in titer after the first titration is doubtless due also to loss of virus by defecation.

If the preceding explanation of an increase in titer be not acceptable, it is necessary to postulate (*a*) gross differences in technique between experiments (perhaps errors in dilution), or (*b*) differences in susceptibility of test animals, or (*c*) wide variation in the amount of virus originally ingested by the mosquitoes, or (*d*) actual multiplication of virus, or (*e*) a biological change in the virus, rendering it more virulent. Injection of mosquitoes at any interval after a meal on infectious blood produces yellow fever. This fact has been amply verified by many workers and has been interpreted as ruling out a life cycle of the virus in mosquitoes. The possibility remains that part of the virus undergoes a cyclical change, or that all undergoes such a change, but at different rates or at different periods, so that at any given time there is some infective virus present. These are theoretical considerations lacking experimental evidence.

In spite of the shortcomings in technique, the experiments indicate that probably some actual loss of virus occurred soon after the infective blood meal. It seems certain that at no time was there present in the mosquitoes a greater amount of virus than the quantity originally ingested.

Spencer and Parker (6) showed that fresh blood activated, or increased the virulence of, Rocky Mountain spotted fever virus in fasting ticks; it is possible that in their experiments the virus (*Rickettsia*) multiplied also.

Dyer, Workman, Ceder, Badger, and Rumreich (7) proved to their satisfaction that the virus of endemic typhus undergoes an enormous multiplication in infected fleas (*Xenopsylla cheopis*). It is probable that all *Rickettsiae* multiply in the arthropod hosts to which they are adapted. Microscopical studies support this view. The behavior of yellow fever virus in the stegomyia mosquito illustrates once more the marked differences between a filterable virus *sensu restricto* and *Rickettsiae*.

Huff (8) has amplified the old classification of insect transmission of disease ("biological" and "mechanical") into four categories: cyclo-propagative, cyclo-developmental, propagative, and mechanical. The behavior of yellow fever virus necessitates still another group, because apparently neither propagation nor cyclical change takes place in the mosquito host; however, an incubation period is obligatory. Evidently migration to the salivary glands occurs during this period. Perhaps this type of transmission might be called "delayed mechanical."

The titer of 1:1,000,000,000 obtained in the first experiment both of Series II and of Series III, when mosquitoes containing freshly ingested blood-virus were used, confirms the work of Bauer (9). The latter produced yellow fever in *rhesus* monkeys with comparable dilutions of infectious blood.

These experiments prove that a stegomyia mosquito may ingest from 1 to 2 million lethal doses of yellow fever virus. An insect which has reached the stage of infectivity may still contain in its body 20,000, or more, lethal doses of virus.

Only two monkeys among survivors of all experiments became immune without showing a fever at some time during the period of observation. Monkey II 1 q had received an injection of mosquito dilution 1:500,000,000; Monkey III 4 j had received a dilution of 1:10,000,000. On the other hand, many animals died of typical yellow fever without having had a demonstrable febrile reaction (104°F., or higher); it is quite possible that transient fevers occurred at night in some of these monkeys.

SUMMARY

Titrations were made of yellow fever virus in stegomyia mosquitoes, using *rhesus* monkeys as test animals. It was found that:

(*a*) The average mosquito immediately after engorging on highly infectious blood contained between 1 and 2 million lethal doses of virus. The titer of freshly ingested blood was as high as 1 billion lethal doses of virus per cubic centimeter.

(*b*) During the fortnight succeeding a meal on infectious blood there occurred a reduction of titratable virus to not more than 1 per cent of that present in the freshly fed insects.

(*c*) The titer was somewhat higher at later periods. This rise in titer signified possibly not a multiplication, but merely an increase of extracellular virus and of that easily freed by grinding to a titratable form.

(*d*) At no later stage did the quantity of titratable virus equal that demonstrable in freshly fed insects.

Comment by Hugo Muench

In experiments such as the foregoing it is probably best to use as end-point the dilution at which one-half the animals die or are affected. In this way the entire group of animals used at different dilutions may in a sense be regarded as one sample, on the assumption that those living at a given dilution would have lived at a lower one, and *vice versa*. This method is a recognized statistical procedure and has the advantage of making the end-point a great deal less variable. The results of using the 50 per cent end-point are given below.

50 Per Cent Mortality

Series I	
Experiment 1	1:1,800,000
Experiment 2	1: 9,000
Experiment 3	1: 1,800
Experiment 4	1: 63,000
Series II	
Experiment 1	1:170,000,000
Experiment 2	1: 500,000
Experiment 3	1: 9,500,000
Experiment 4	1: 10,000,000
Experiment 5	1: 2,500,000
Experiment 6	1: 2,500,000

Series III	
Experiment 1	1:700,000,000
Experiment 2	1: 7,000,000
Experiment 3	1: 28,000,000
Experiment 4	1: 28,000,000

On comparing the figures in this tabulation with those of the text, the following main differences will be seen: (1) when the 50 per cent mortality point is used, the secondary rise is not nearly so pronounced, although it is uniformly present and is probably real. (2) The difference between the two sets of mosquitoes in Series II (those receiving and those not receiving additional blood meals) almost disappears. It is doubtful whether there is any such real difference. This would agree with the findings of the authors in Series III.

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