INTERFERENCE BETWEEN VIRUSES IN TISSUE CULTURE*

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The observations of many workers have amply shown that infection of a host by one virus may, under certain conditions, afford temporary protection against infection by another. The antagonistic action between the two viruses, which results in the dominance of one and confers on the host a transitory resistance to superinfection with the other, is generally referred to as "interference."

This phenomenon was first described for plant viruses. McKinney (1) in 1929 reported that the yellow-mosaic tobacco virus did not multiply in plants previously infected with the related common-mosaic virus. Interference between various plant viruses has been studied by many others since then, and the work has been summarized recently by Price (2).

The existence of interference between animal viruses was reported in 1935 by Hoskins (3) and by Magrassi (4). Hoskins found that monkeys infected with a neurotropic strain of yellow fever virus were protected against fatal infection with a pantropic strain, and Magrassi reported that rabbits infected with non-encephalitogenic strains of herpes virus were resistant to cerebral inoculation of encephalitogenic strains. Similar antagonistic effects in animal hosts have since been described between the viruses of Rift Valley fever and yellow fever (5), lymphocytic choriomeningitis and poliomyelitis (6–8), murine and simian poliomyelitis (9–12), virus III and Shope fibroma (13), mouse encephalomyelitis and Western equine encephalomyelitis (14), and Eastern and Western equine encephalomyelitis (15, 16).

Henle and Henle (17) found that inactive influenza virus can interfere with the growth of active virus in the chick embryo, and Ziegler and his associates (18, 19) also using chick embryos, have shown the occurrence of a reciprocal interfering effect between type A, B, and swine influenza viruses.

Andrewes (20), using pneumotropic and neurotropic strains of influenza A virus, was able to demonstrate the occurrence of reciprocal interference in tissue cultures.

Because of the numerous difficulties surrounding the demonstration and study of interference in animal hosts, the feasibility of using tissue cultures for these purposes was investigated further. The present report deals with observations made on *in vitro* interference between a number of viruses.

Methods

Viruses.—The viruses of yellow fever, West Nile disease, Venezuelan equine encephalomyelitis, and influenza A were used.

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The yellow fever virus was represented by two strains, the classic Asibi and its tissue culture derivative, 17DD High. Cultures of the Asibi strain were initiated with suspensions of brains from mice infected with 39th *rhesus* monkey passage serum virus. The 17DD virus is a tissue culture substrain derived from the 195th subculture of the original 17D strain of Theiler and Smith (21); this substrain, when 305 or more culture passages removed from the parent Asibi virus, is called 17DD High because of certain changes in the characteristics of the virus at this culture level (22).

The West Nile virus (23), strain M-956, was started in cultures with the use of 139th mouse brain passage virus.

The Venezuelan equine encephalomyelitis virus employed represented the original virus described by Kubes and Rios (24) and by Beck and Wyckoff (25); its passage history is uncertain. Mouse brain passage virus was used to initiate the tissue culture passages.

The PR8 strain (26) of influenza A virus had undergone 337 mouse passages and 7 chick embryo passages prior to cultivation *in vitro*, which was begun with the use of pooled allantoic fluids from the 7th egg passage.

Tissue Cultures.—Cultures were made in 50 ml. Erlenmeyer flasks closed with cork stoppers covered with tin foil. The fluid portion of the medium consisted of 4.0 ml. of menstruum; for the cultivation of influenza virus, plain Tyrode's solution was used, while for the other viruses Tyrode's solution containing 10 per cent of normal human serum was used.

For routine passages the tissue component varied with the virus, but was always used in the proportion of 1 drop (0.1 gm.) of tissue mince to 4.0 ml. of fluid, except in the cultivation of influenza virus, where larger amounts (0.2 to 0.4 gm.) of tissue were required.[1] Minced whole mouse embryo was used for propagation of the Asibi virus, while the influenza virus was carried in cultures containing minced 12- to 13-day-old chick embryos from which the eyes, beak, and legs had been removed, and the 17DD, West Nile, and Venezuelan equine encephalomyelitis viruses were maintained in cultures containing minced 8- to 10-day-old embryos from which the central nervous system had been cut away. The viruses were maintained by transfer of 0.5 ml. of fluid from infected cultures to freshly prepared media; influenza virus was passaged at 2 day intervals, other viruses at 3 to 4 day intervals. All cultures were incubated at 37°C.

In the interference experiments, the tissue component was invariably minced 8- to 10-day-old chick embryos without the central nervous system.

Interference Experiments.—Freshly prepared flasks of culture medium were divided into three equal lots. Two lots were inoculated with 0.5 ml. amounts of centrifuged pooled supernatant fluid from a previous culture passage of the required virus, the remaining lot was left uninoculated, and all were placed in an incubator at 37°. After 24 hours, 0.5 ml. amounts of centrifuged pooled supernatant fluid from tissue cultures of the second virus were introduced into the uninoculated cultures and into one lot of the cultures inoculated the day before. The cultures were incubated for an additional 2 days when either the first or second inoculum contained influenza virus, and for an additional 3 days when the inocula contained viruses other than influenza.

Because of the difficulty in securing large amounts of human serum free of antibodies to the influenza virus, the use of serum was avoided in work with this virus; the virus was carried in

[1] A number of attempts made in this laboratory to cultivate influenza virus by the usual technique, employing minced chick tissue and Tyrode's solution (20, 27), were negative. Changes in the composition of the fluid component, such as addition of nutrient broth to the Tyrode's solution, or its substitution by Simm's solution, did not influence the results. Finally, use of amounts of minced chick tissue larger than the amount used as routine in the cultivation of other viruses, resulted in establishment of tissue culture strains.

serum-free culture media and in the interference experiments only serum-free cultures were used, regardless of whether the influenza virus was added to the cultures in the first or in the second inoculum.

The amount of active virus seeded into the cultures was determined by titration in albino Swiss mice of 28 to 40 days of age, which for convenience will be referred to as young adult mice. Influenza virus was titrated by the intranasal route, the others by the intracerebral route. Serial tenfold dilutions were made in Tyrode's solution (influenza virus) or in serum–Tyrode's solution, and each dilution was inoculated into a group of 6 mice. The titer of the virus was computed on the basis of the 50 per cent mortality end point (28), from which the number of intranasal or intracerebral LD_{50} of virus in the 0.5 ml. of inoculum was calculated.

To detect the occurrence of interference in the cultures, mice were used as indicator animals. In the case of the neurotropic viruses, advantage was taken of the differences in susceptibility of mice, depending on the age of the animal, to extraneural inoculation of the viruses employed (29). By using mice young enough to be susceptible to the second virus, but old enough to resist infection with the first, or interfering, virus, it was possible to determine the extent to which interference with the growth of the second virus had occurred. Serial fourfold dilutions of the centrifuged supernatant fluid from each test and control culture were made in Tyrode's or serum–Tyrode's solution and inoculated subcutaneously or intraperitoneally into mice of selected ages. Because of the variation in the age of the animals and the extraneural routes of inoculation used, specific information is given with each experiment. Each dilution was inoculated into a group of 6 mice, using 0.03 ml. per animal; the same material was usually also inoculated intracerebrally into groups of 6 mice 28 to 40 days of age. Influenza virus was quantitated by intranasal inoculation of 28- to 40-day-old mice with 0.03 ml. of culture dilution, using 6 animals per dilution.

Mice inoculated with preparations containing influenza virus were observed for 10 days, at the end of which time the number of dead mice was used to compute the LD_{50} titer; mice alive on the 10th day were killed and the total number showing one plus or more consolidation of the lungs was used to calculate the 50 per cent infectivity end point, or ID_{50}. Mice inoculated with other viruses were observed during a 21 day period, and the LD_{50} titer was computed from specific deaths occurring within this interval.

In addition, the average survival times of the inoculated mice were calculated according to the method devised by Kerr and described by Bugher (30). Comparison of the average survival time of mice inoculated with doubly infected cultures and the average survival time of mice inoculated with control cultures provided a useful auxiliary means of determining to which of the two viruses infection of the animal was due.

Finally, on some occasions specific immune sera were used to identify the virus present in the brains of mice succumbing to infection with material from dually infected cultures. Sick mice were killed, and their brains were removed and made into a 20 per cent suspension in 10 per cent normal human serum–Tyrode's solution. The supernatant obtained after centrifugation in an angle head for 15 minutes at 1,500 R.P.M. was used to prepare a series of tenfold dilutions; an aliquot of each of the desired dilutions was mixed with an equal amount of normal or immune rabbit serum and inoculated subcutaneously into 3-day-old mice or intraperitoneally into older animals (31).

RESULTS

Interference of 17DD High Strain of Yellow Fever Virus with the Growth of the Asibi Strain

Two experiments were done to determine whether tissue cultures containing the 17DD High strain would permit the Asibi strain to multiply. Three-day-

old mice were used as indicator animals, since it has been observed that the 17DD High strain, unlike the Asibi (32), is incapable of producing fatal infections in mice of this age on subcutaneous inoculation.

The results of both experiments are given in Table I. In Experiment 1, none of the 3-day-old mice succumbed to inoculation of cultures infected with the

TABLE I

Yellow Fever Virus. Interference of 17DD High Strain with Growth of the Asibi Strain

Experiment No.	Culture flask	Inoculation of cultures: First inoculum* Virus	First inoculum* LD_{50}	Second inoculum* (24 hrs. after first inoculation) Virus	Second inoculum* LD_{50}	LD_{50} virus titer of cultures (72 hrs. after second inoculation): 3-day-old mice (Subcutaneous route)		Young adult mice (intracerebral route)	
						mean			
1	1a	17DD	$10^{5.9}$	None		0‡			
	2a					0			
	3a					0			
	4a					0			
	5a	17DD	$10^{5.9}$	Asibi	$10^{5.2}$	$10^{-0.80}$			
	6a					$10^{-0.70}$	$10^{-0.75}$		
	7a					$10^{-0.85}$			
	8a					$10^{-0.90}$			
	9a	None		Asibi	$10^{5.2}$	$10^{-2.35}$			
	10a					$10^{-2.90}$	$10^{-2.80}$		
	11a					$10^{-3.00}$	or higher		
	12a					$>10^{-3.00}$			
								mean	
	1b	17DD	$10^{5.0}$	None		0		$10^{-3.00}$	
	2b					0		$10^{-3.30}$	$10^{-3.25}$
	3b					0		$10^{-3.45}$	
	4b	17DD	$10^{5.0}$	Asibi	$10^{5.7}$	0		$10^{-3.95}$	
	5b					0		$10^{-4.15}$	$10^{-4.10}$
	6b					0		$10^{-4.20}$	
	7b	None		Asibi	$10^{5.7}$	$10^{-2.00}$		$10^{-5.10}$	
	8b					$10^{-2.15}$	$10^{-2.15}$	$10^{-4.70}$	$10^{-4.90}$
	9b					$10^{-2.25}$		$10^{-4.95}$	

* In Experiment 1, 641st passage 17DD and 14th passage Asibi virus were used; in Experiment 2, 640th passage 17DD and 27th passage Asibi were used.

‡ The symbol 0 indicates all mice inoculated with undiluted culture supernatant survived.

17DD virus alone, whereas mice receiving the Asibi cultures alone were killed by dilutions as great as 10^{-3} or more. Cultures inoculated first with the 17DD and subsequently with the Asibi virus showed the presence of only minimal amounts of Asibi virus, as is indicated by the low LD_{50} titers.

Comparison of the mean titers shows that the 17DD High strain of virus interfered with the growth of the Asibi virus to such an extent that it reached only about 1 per cent of the concentration that it attained when growing alone.

The results were checked in a second experiment, shown in Table I. It will be observed that the Asibi virus grew in the control cultures, as shown by their infectivity for 3-day-old mice, whereas its growth in cultures previously inoculated with 17DD virus was completely suppressed; the virus present in the doubly inoculated cultures presumably represented the 17DD strain, inasmuch as the cultures were infectious by the cerebral route, but not by the subcutaneous route. Comparison of the average survival times of mice inoculated with the three different series of cultures pointed to a similar conclusion. The average survival times of mice inoculated with 17DD and 17DD-Asibi cultures

TABLE II

Interference of 17DD High Strain of Yellow Fever Virus with the Growth of West Nile Virus

Experiment No.	Culture flask	Inoculation of cultures: First inoculum* Virus	First inoculum* LD_{50}	Second inoculum* (24 hrs. after first inoculation) Virus	Second inoculum* LD_{50}	LD_{50} virus titer of cultures (72 hrs. after second inoculation): 3-day-old mice (subcutaneous route)		Young adult mice (intracerebral route)	
								mean	
1	1	17DD	$10^{4.5}$	None		0		$10^{-2.10}$	
	2					0		$10^{-2.85}$	$10^{-2.95}$
	3					0		$10^{-3.40}$	
	4					0		$10^{-3.40}$	
	5	17DD	$10^{4.5}$	W. Nile	$10^{3.4}$	0		$10^{-2.35}$	
	6					0		$10^{-2.75}$	$10^{-2.80}$
	7					0		$10^{-2.90}$	
	8					0		$10^{-3.25}$	
						mean			
	9	None		W. Nile	$10^{3.4}$	$10^{-3.90}$		$10^{-3.80}$	
	10					$10^{-4.05}$	$10^{-4.20}$	$10^{-4.45}$	$10^{-4.25}$
	11					$10^{-4.30}$		$10^{-4.25}$	
	12					$10^{-4.55}$		$10^{-4.50}$	

* The viruses used were 656th passage 17DD and 10th passage West Nile.

were similar and in both cases were longer than the average survival time of mice which received the Asibi control cultures.

The results of both experiments, therefore, showed that the 17DD High strain was able to suppress completely, or almost completely, the growth of the parent Asibi strain.

Interference of Yellow Fever Virus with the Growth of West Nile Virus

The possibility that the 17DD High strain of yellow fever virus might exert as marked an interfering effect upon the growth of serologically unrelated neurotropic viruses as it did upon the growth of the related Asibi strain of yellow fever virus was next investigated. The West Nile virus was used in the first of these experiments.

The results of one experiment are presented in Table II. Titration of the cultures in 3-day-old mice showed that while the West Nile virus had multiplied readily in the control flasks, its growth was completely suppressed in cultures previously inoculated with the 17DD virus. Evidence of interference was also obtained from the titration end points computed from the results of intracerebral inoculation of the cultures: as is shown in Table II, the average LD_{50} titers of the virus in the 17DD and 17DD–West Nile cultures were not only lower than the LD_{50} titer of the West Nile cultures, but were practically identical, so that the infectivity of the 17DD–West Nile cultures was considered due to the 17DD virus. Calculation of the average survival times supported this assumption, since the average survival times of mice inoculated with 17DD cultures were indistinguishable from those of mice inoculated with the 17DD–West Nile cultures, and both in turn were longer than the average survival times of mice inoculated with the West Nile cultures. Finally, neutralization tests done by the subcutaneous route in 3-day-old mice and by the intracerebral route in older mice showed that only the yellow fever virus was present in the brains of mice succumbing to infection after cerebral inoculation of the 17DD–West Nile cultures.

Interference of Yellow Fever Virus with the Growth of Venezuelan Equine Encephalomyelitis Virus

Experiments on the ability of the 17DD yellow fever virus to interfere with the growth of the Venezuelan equine encephalomyelitis virus are summarized in Table III.

In the first two experiments, fixed quantities of each of the viruses were used. It will be observed that in both experiments the 17DD virus characteristically failed to kill 3-day-old mice, although the presence of active virus in the cultures was attested by the results of intracerebral inoculation in young adult mice. Cultures infected with both 17DD and Venezuelan viruses, on the other hand, produced fatal infections in 3-day-old mice, showing that the latter virus was present; however, only the lower dilutions of the cultures were lethal, so that comparatively little, if any, multiplication of virus in the original inoculum had occurred. Comparison of the mean LD_{50} titers shows that marked interference with the growth of the Venezuelan virus had occurred; expressed on a percentage basis, the 17DD virus interfered with the multiplication of the Venezuelan virus to such an extent that the doubly infected cultures contained only approximately 0.6 per cent and 0.2 per cent (Experiments 1 and 2, respectively) of the amount of Venezuelan virus present in the controls.

Evidence of interference, although less striking, was obtained from the results of intracerebral inoculation of mice with the cultures. The lower LD_{50} titers of the doubly infected cultures as compared with the Venezuelan virus control cultures indicated only that the infectivity of these cultures was due either to

the 17DD virus or to Venezuelan virus present in lower concentrations than in the control cultures. In both Experiments 1 and 2, however, the average survival times of mice inoculated with the doubly infected cultures were significantly shorter than those of mice inoculated with the 17DD control cultures and were approximately the same as those of mice inoculated with the Venezuelan virus cultures. The average survival times, therefore, indicated that deaths following inoculation of the 17DD-Venezuelan virus cultures were due to the Venezuelan rather than to the 17DD component, and the lower LD_{50} titers of these cultures, compared with the Venezuelan virus controls, were interpreted as pointing to some suppression of growth of this virus.

In view of the marked interfering effect of 17DD virus on the Venezuelan virus, even when the latter was introduced into cultures in large amounts, a third experiment was done using graded doses of the Venezuelan virus to see whether complete suppression of its growth could be effected. The results are shown under Experiment 3 in Table III.

In cultures previously infected with the 17DD virus, growth of the Venezuelan virus occurred only in cultures 5c and 6c, which had been inoculated with the larger amounts ($10^{6.7}$ and $10^{4.7}$ mouse LD_{50} respectively) of this virus, and was completely suppressed in cultures 7c and 8c, which had received the smaller doses ($10^{2.7}$ and $10^{0.7}$ LD_{50} respectively).

Comparison of the LD_{50} titer of 17DD-Venezuelan culture 5c with its corresponding Venezuelan virus control culture 9c, both inoculated with $10^{6.7}$ LD_{50} of the Venezuelan virus, shows that the latter culture contained roughly 20 times more of this virus than did the former. When cultures were inoculated with 100 times less Venezuelan virus, the difference was marked; comparison of the LD_{50} titers of 17DD-Venezuelan culture 6c with its Venezuelan virus control 10c, both of which originally received $10^{4.7}$ LD_{50} of the Venezuelan virus, reveals that in the absence of the yellow fever virus the Venezuelan virus attained a titer 28,000 times higher than it did in the presence of this virus.

Decreasing the amount of Venezuelan virus in the culture inoculum by another hundredfold or more resulted in complete suppression of its growth. The absence of Venezuelan virus in the doubly inoculated cultures 7c and 8c cannot be ascribed to insufficient virus in the inoculum, since control cultures 11c and 12c received correspondingly identical amounts of virus and the final titer attained was higher than in controls inoculated with larger amounts.

With regard to the results in mice inoculated intracerebrally, the average survival time of mice receiving cultures 7c and 8c, in which the growth of the Venezuelan virus had been suppressed, approximated the average survival time of mice receiving the 17DD cultures, while the average survival time of mice inoculated with the doubly infected cultures 5c and 6c, approximated the average survival time of the Venezuelan virus cultures. The intracerebral LD_{50} titers of the 17DD-Venezuelan cultures 5c and 6c were 100 and 3,000

TABLE III

Interference of 17DD High Strain of Yellow Fever Virus with Growth of the Venezuelan Equine Encephalomyelitis Virus

Experiment No.	Culture flask	Inoculation of cultures: First inoculum* Virus	First inoculum* LD_{50}	Second inoculum* (24 hrs. after first inoculation) Virus	Second inoculum* LD_{50}	LD_{50} virus titer of cultures (72 hrs. after second inoculation): 3-day-old mice (subcutaneous route)	mean	Young adult mice (intracerebral route)	mean
1	1a	17DD	$10^{5.1}$	None		0		$10^{-2.85}$	$10^{-2.35}$
	2a					0		$10^{-3.00}$	
	3a					0		$10^{-3.75}$	
	4a					0		$10^{-3.85}$	
	5a	17DD	$10^{5.1}$	Ven. e. e.	$10^{6.7}$	$10^{-1.40}$	$10^{-2.55}$	$10^{-2.50}$	$10^{-2.85}$
	6a					$10^{-1.80}$		$10^{-2.20}$	
	7a					$10^{-2.55}$		$10^{-2.20}$	
	8a					$10^{-4.45}$		$10^{-3.45}$	
	9a	None		Ven. e. e.	$10^{6.7}$	$10^{-4.65}$	$>10^{-4.75}$	$10^{-3.80}$	$>10^{-4.55}$
	10a					$>10^{-4.80}$		$>10^{-4.80}$	
	11a					$>10^{-4.80}$		$>10^{-4.80}$	
	12a					$>10^{-4.80}$		$>10^{-4.80}$	
2	1b	17DD	$10^{4.7}$	None		0		$10^{-2.55}$	$10^{-3.75}$
	2b					0		$10^{-3.70}$	
	3b					0		$10^{-3.85}$	
	4b					0		$10^{-3.85}$	
	5b	17DD	$10^{4.7}$	Ven. e. e.	$10^{5.1}$	$10^{-2.25}$	$10^{-2.95}$	$10^{-2.55}$	$10^{-3.40}$
	6b					$10^{-2.55}$		$10^{-4.20}$	
	7b					$10^{-3.50}$		$10^{-3.35}$	
	8b					$10^{-3.60}$		$10^{-3.45}$	
	9b	None		Ven. e. e.	$10^{5.1}$	$10^{-5.05}$	$10^{-5.60}$	$10^{-4.45}$	$10^{-4.90}$
	10b					$10^{-5.40}$		$10^{-5.05}$	
	11b					$10^{-5.70}$		$10^{-4.80}$	
	12b					$10^{-6.20}$		$10^{-4.80}$	
3	1c	17DD	$10^{5.0}$	None		0		$10^{-2.65}$	$10^{-3.00}$
	2c					0		$10^{-2.80}$	
	3c					0		$10^{-3.30}$	
	4c					0		$10^{-3.30}$	
	5c	17DD	$10^{5.0}$	Ven. e. e.	$10^{6.7}$	$10^{-4.00}$	$10^{-1.45}$	$10^{-3.25}$	$10^{-2.75}$
	6c				$10^{4.7}$	$10^{-1.80}$		$10^{-2.50}$	
	7c				$10^{3.7}$	0		$10^{-2.40}$	
	8c				$10^{0.7}$	0		$10^{-2.85}$	
	9c	None		Ven. e. e.	$10^{6.7}$	$10^{-5.25}$	$10^{-6.20}$	$10^{-5.25}$	$10^{-6.10}$
	10c				$10^{4.7}$	$10^{-6.25}$		$10^{-6.00}$	
	11c				$10^{3.7}$	$>10^{-6.60}$		$>10^{-6.6}$	
	12c				$10^{0.7}$	$>10^{-6.60}$		$>10^{-6.60}$	

* The viruses used were 647th passage 17DD and 6th passage Venezuelan equine encephalomyelitis in Experiment 1; 651st passage 17DD and 14th passage Venezuelan equine encephalomyelitis in Experiment 2; 647th passage 17DD and 34th passage Venezuelan equine encephalomyelitis in Experiment 3.

times lower, respectively, than the corresponding Venezuelan control cultures 9c and 10c, showing not only that interference had occurred, but also that the effect is more pronounced if smaller amounts of the second virus are used.

In summary, it was found that the 17DD High strain of yellow fever virus could interfere with the growth of the serologically unrelated Venezuelan equine encephalomyelitis virus, and that the degree of interference depended upon the quantity of Venezuelan virus used.

Interference of West Nile Virus with the Growth of Venezuelan Equine Encephalomyelitis Virus

Since the 17DD High strain of yellow fever virus interfered with the growth of both the West Nile and Venezuelan equine encephalomyelitis virus, it appeared of interest to see whether either of the latter viruses could interfere with the growth of the other. Inasmuch as mice as old as 200 days are highly susceptible to peripheral inoculation of the Venezuelan virus (29), the experiment had to be limited to the effect of West Nile virus on the growth of the Venezuelan virus. Thirty-nine-day-old mice were chosen as indicator animals, since mice of this age are highly susceptible to intraperitoneal inoculation of low passage tissue culture Venezuelan equine encephalomyelitis virus (33), but insusceptible to culture passage West Nile virus (33).

The results of the first experiment are given in Table IV. It will be seen that the West Nile cultures were lethal for mice by the cerebral, but not by the peritoneal, route; the Venezuelan virus cultures, on the other hand, were lethal by both routes. The death of mice receiving the doubly inoculated cultures intraperitoneally must therefore be ascribed to the Venezuelan virus. However, the LD_{50} titers of the West Nile–Venezuelan equine encephalomyelitis cultures were all somewhat lower than those of the Venezuelan control cultures, so that some interfering action, presumably, must have been exerted by the West Nile virus. The difference in the mean LD_{50} titers shows that the control cultures contained 25 times more Venezuelan virus than did the dually infected cultures; since the titrations were based on the use of serial fourfold dilutions, this difference in the mean titers may represent a true difference, and not experimental error.

The average survival time indicated that mice inoculated intracerebrally with the dually infected cultures succumbed to the action of the Venezuelan virus rather than to the West Nile virus, although the brains of such mice contained both viruses as shown by neutralization tests. The titers of the cultures inoculated with both viruses were consistently lower than the titers of the Venezuelan control cultures, but the differences were too small to be interpreted unequivocally as due to interference.

The suggestive results made it seem likely that more clear cut results might be obtained by the use of smaller amounts of the Venezuelan virus, and the ex-

periment was repeated using graded doses of this virus. The results are shown under Experiment 2 in Table IV. The West Nile cultures characteristically were non-lethal for mice by the intraperitoneal route, although they contained active virus as shown by cerebral inoculation. Inoculation of a West Nile culture (flask 5b) with $10^{5.8}$ LD_{50} of the Venezuelan virus permitted growth of

TABLE IV

Interference of West Nile Virus with Growth of Venezuelan Equine Encephalomyelitis Virus

Experiment No.	Culture flask	Inoculation of cultures: First inoculum* Virus	First inoculum* LD_{50}	Second inoculum* (24 hrs. after first inoculation) Virus	Second inoculum* LD_{50}	LD_{50} virus titer of cultures (72 hrs. after second inoculation): 39-day-old mice (intraperitoneal route)	mean	Young adult mice (intracerebral route)	mean
1	1a	W. Nile	$10^{3.8}$	None		0		$10^{-2.55}$	
	2a					0		$10^{-2.60}$	
	3a					0		$10^{-2.20}$	$10^{-2.80}$
	4a					0		$10^{-2.60}$	
	5a	W. Nile	$10^{3.8}$	Ven. e. e.	$10^{5.8}$	$10^{-1.65}$		$10^{-2.90}$	
	6a					$10^{-2.10}$		$10^{-3.60}$	
	7a					$10^{-2.75}$	$10^{-2.45}$	$10^{-4.00}$	$10^{-3.65}$
	8a					$10^{-3.40}$		$10^{-4.15}$	
	9a	None		Ven. e. e.	$10^{5.8}$	$10^{-3.35}$		$10^{-4.40}$	
	10a					$10^{-3.35}$		$10^{-4.55}$	
	11a					$10^{-4.15}$	$10^{-3.85}$	$10^{-4.95}$	$10^{-4.75}$
	12a					$10^{-4.60}$		$10^{-5.15}$	
2	1b	W. Nile	$10^{4.0}$	None		0		$10^{-3.80}$	
	2b					0		$10^{-3.00}$	
	3b					0		$10^{-3.10}$	$10^{-3.05}$
	4b					0		$10^{-3.30}$	
	5b	W. Nile	$10^{4.0}$	Ven. e. e.	$10^{5.8}$	$10^{-2.25}$		$10^{-3.50}$	
	6b				$10^{4.8}$	0		$10^{-3.10}$	
	7b				$10^{1.8}$	0	$10^{-0.55}$	$10^{-3.10}$	$10^{-3.15}$
	8b				$10^{0.8}$	0		$10^{-2.95}$	
	9b	None		Ven. e. e.	$10^{5.8}$	$10^{-4.15}$		$10^{-5.30}$	
	10b				$10^{4.8}$	$10^{-5.00}$		$10^{-5.20}$	
	11b				$10^{1.8}$	$10^{-5.10}$	$10^{-3.55}$	$10^{-5.25}$	$10^{-3.95}$
	12b				$10^{0.8}$	0		0	

* Viruses used were 18th passage West Nile and 2nd passage Venezuelan equine encephalomyelitis in Experiment 1 and 25th passage West Nile and 2nd passage Venezuelan equine encephalomyelitis in Experiment 2.

the latter, as evidenced by the lethal effect of this culture on intraperitoneal inoculation. Some suppression of growth of the Venezuelan virus occurred, however, since comparison of the LD_{50} titers shows that this culture contained only 1 per cent as much Venezuelan virus as did its corresponding control culture (flask 9b).

Inoculation of West Nile cultures with smaller quantities ($10^{3.8}$ or $10^{1.8}$ LD_{50})

of Venezuelan virus resulted in complete suppression of growth of this virus (*cf.* culture flasks 6b and 7b with control cultures 10b and 11b.).

The average survival time of mice inoculated intracerebrally with West Nile–Venezuelan equine encephalomyelitis cultures 6b to 8b was longer that the average survival time of mice inoculated with Venezuelan control cultures and the same as that of mice inoculated with the West Nile control cultures, pointing to the presence of West Nile virus and the absence of Venezuelan virus in the dually inoculated cultures. This was confirmed by neutralization tests done with the brains of mice which died following inoculation of West Nile–Venezuelan cultures 6b and 7b: neutralization was effected only with West Nile immune serum.

These experiments show that the West Nile virus can interfere with the growth of the Venezuelan virus in tissue cultures and that, as was also shown with the yellow fever and Venezuelan viruses, quantitative considerations are involved in the interference phenomenon.

Interference of Yellow Fever Virus with the Growth of Influenza A Virus

The preceding experiments were concerned with the detection of interference between viruses which, although unrelated serologically, possess, at least in the mouse, a common tissue tropism. Although it is recognized that the cells utilized by a virus for growth *in vivo* may be quite different from those utilized *in vitro*, it was considered worthwhile to investigate whether interference can occur between two viruses whose antigenic constitution and tissue affinities (in the mouse) are distinct.

The influenza A virus, PR8 strain, was chosen because its ability to kill and to produce distinctive pulmonary lesions on intranasal inoculation afforded a means of differentiating it in mice from a virus like that of yellow fever, which neither kills nor produces pulmonary changes when administered by the nasal route. Several experiments on reciprocal interference between these two viruses were done. The results are given in Table V.

The first two experiments were concerned with the effect of 17DD High strain yellow fever virus on the growth of the influenza virus. Table V shows that in both Experiments 1 and 2, mice inoculated intranasally with cultures containing only influenza virus died, or survived with gross evidence of pulmonary consolidation present when killed on the 10th postinoculation day, while mice similarly inoculated with cultures of yellow fever virus alone survived, and when killed showed no pulmonary lesions. In Experiment 1, cultures inoculated first with the yellow fever virus and subsequently with influenza virus by the intranasal route neither killed mice nor produced pulmonary lesions. In Experiment 2 the dually inoculated cultures also failed to kill, but traces of influenza virus were present in three of the four cultures, as evidenced by the presence of minimal lung lesions in animals inoculated with lowest culture dilutions.

TABLE V

Experiments on Reciprocal Interference between 17DD High Strain Yellow Fever Virus and PR8 Influenza A Virus

Experiment No.	Culture flask	Inoculation of cultures: First inoculum* Virus	First inoculum* LD_{50}	Second inoculum* (24 hrs. after first inoculation) Virus	Second inoculum* LD_{50}	Virus titer of cultures (48 hrs. after second inoculation): Mice inoculated by (Intranasal route) LD_{50} titer		(Intranasal route) ID_{50} titer		(Intracerebral route) LD_{50} titer	
						mean		*mean*		*mean*	
1	1a	17DD	$10^{3.8}$	None		0		0		$10^{-2.70}$	
	2a					0		0		$10^{-2.85}$	
	3a					0		0		$10^{-3.15}$	$10^{-2.95}$
	4a					0		0		$10^{-3.20}$	
	5a	17DD	$10^{3.8}$	PR8	$10^{2.5}$	0		0		$10^{-2.75}$	
	6a					0		0		$10^{-3.00}$	$10^{-2.95}$
	7a					0		0		$10^{-3.50}$	
	8a					0		0		$10^{-3.70}$	
	9a	None		PR8	$10^{2.5}$	$10^{-3.15}$		$10^{-4.40}$		0	
	10a					$10^{-3.15}$	$10^{-2.55}$	$10^{-5.10}$	$10^{-4.85}$	0	
	11a					$10^{-3.70}$		$10^{-5.40}$		0	
	12a					$10^{-4.30}$		$10^{-4.55}$		0	
2	1b	17DD	$10^{3.8}$	None		0		0		$10^{-3.60}$	
	2b					0		0		$10^{-3.70}$	$10^{-4.00}$
	3b					0		0		$10^{-4.00}$	
	4b					0		0		$10^{-4.55}$	
	5b	17DD	$10^{3.8}$	PR8	$10^{4.3}$	0		0		$10^{-3.35}$	
	6b					0		$10^{-0.30}$	$10^{-0.40}$	$10^{-4.60}$	$10^{-4.15}$
	7b					0		$10^{-0.45}$	or less	$10^{-3.90}$	
	8b					0		$10^{-0.95}$		$10^{-4.20}$	
	9b	None		PR8	$10^{4.3}$	$10^{-2.85}$		$10^{-4.95}$		0	
	10b					$10^{-2.95}$	$10^{-3.05}$	$10^{-5.10}$	$10^{-4.85}$	0	
	11b					$10^{-3.15}$		$10^{-4.90}$		0	
	12b					$10^{-3.25}$		$10^{-4.40}$		0	
3	1c	PR8	$10^{4.2}$	None		$10^{-2.35}$		$10^{-3.80}$		0	
	2c					$10^{-2.80}$	$10^{-2.85}$	$10^{-4.50}$	$10^{-4.20}$	0	
	3c					$10^{-3.00}$		$10^{-3.85}$		0	
	4c					$10^{-3.00}$		$10^{-4.70}$		0	
	5c	PR8	$10^{4.2}$	17DD	$10^{5.0}$	$10^{-2.25}$		$10^{-4.40}$		$>10^{-3.60}$	
	6c					$10^{-2.30}$	$10^{-2.60}$	$10^{-3.20}$	$10^{-4.00}$	$10^{-3.25}$	$10^{-3.40}$
	7c					$10^{-2.50}$		$10^{-4.80}$		$10^{-3.30}$	or higher
	8c					$10^{-3.30}$		$10^{-3.60}$		$10^{-3.45}$	
	9c	None		17DD	$10^{5.0}$	0		0		$10^{-2.75}$	
	10c					0		0		$10^{-3.55}$	$10^{-3.60}$
	11c					0		0		$10^{-3.90}$	
	12c					0		0		$10^{-4.30}$	

* The viruses used were 681st passage 17DD and 14th passage PR8 in Experiment 1; 684th passage 17DD and 20th passage PR8 in Experiment 2; 687th passage 17DD and 23rd passage PR8 in Experiment 3; and 693rd passage 17DD and 33rd passage PR8 in Experiment 4.

TABLE V—*Concluded*

Experiment No.	Culture flask	Inoculation of cultures: First inoculum* Virus	First inoculum* LD_{50}	Second inoculum* (24 hrs. after first inoculation) Virus	Second inoculum* LD_{50}	Virus titer of cultures (48 hrs. after second inoculation): Mice inoculated by (Intranasal route) LD_{50} titer	mean	ID_{50} titer	mean	(Intracerebral route) LD_{50} titer	mean
4	1d	PR8	$10^{3.5}$	None		$10^{-2.50}$	$10^{-2.90}$	$10^{-3.25}$	$10^{-3.85}$	0	
	2d					$10^{-2.85}$		$10^{-3.90}$		0	
	3d					$10^{-2.85}$		$10^{-4.20}$		0	
	4d					$10^{-3.50}$		$10^{-4.05}$		0	
	5d	PR8	$10^{3.5}$	17DD	$10^{3.9}$	$10^{-2.65}$	$10^{-2.05}$	$10^{-3.25}$	$10^{-2.75}$	$10^{-2.70}$	$10^{-2.25}$
	6d				$10^{2.9}$	$10^{-1.60}$		$10^{-2.85}$		$10^{-2.90}$	
	7d				$10^{1.9}$	$10^{-1.95}$		$10^{-2.60}$		$10^{-2.00}$	
	8d				$10^{0.9}$	$10^{-2.00}$		$10^{-2.35}$		$10^{-1.80}$	
	9d	None		17DD	$10^{3.9}$	0		0		$10^{-2.15}$	$10^{-2.50}$
	10d				$10^{2.9}$	0		0		$10^{-2.85}$	
	11d				$10^{1.9}$	0		0		$10^{-2.85}$	
	12d				$10^{0.9}$	0		0		$10^{-2.25}$	

It is evident, therefore, that the yellow fever virus was able to suppress completely, or almost completely, the growth of the influenza A virus.

Since experiments in the reverse direction were practicable because of the difference in tropism of the viruses, the effect of influenza A virus on the growth of yellow fever virus was investigated. Two experiments, numbered 3 and 4 in Table V, were done.

In Experiment 3, in which a fixed, large amount of 17DD virus was added to cultures infected with PR8, no interference with growth was demonstrable, as is shown by the results of intracerebral titration: the mean intracerebral LD_{50} titers of the dually inoculated cultures and the control 17DD cultures were practically the same.

On the assumption that too much 17DD virus had been used, Experiment 4 was set up in which graded amounts of this virus were added to cultures of PR8. Despite the fact that as little as $10^{0.9}$ LD_{50} of yellow fever virus were used, however, its multiplication was not significantly influenced by the influenza virus. Table V shows that the intracerebral LD_{50} titers of the dually infected cultures differed very little from those of the yellow fever control cultures.

In summary, while it was possible under the experimental conditions employed to show that yellow fever virus interfered with the growth of influenza A virus, it was not possible to demonstrate that interference occurs in the reverse direction. In this connection it may be of interest to note that the individual (as well as mean) LD_{50} and ID_{50} titers of influenza virus in the dually inoculated cultures in Experiment 4 were somewhat lower than those of the

influenza control cultures, pointing possibly to a depressing effect exerted by the yellow fever virus despite the fact that it was added to the cultures 24 hours after the influenza virus; *i.e.*, to a reversal of the direction in which interference would normally be expected to go.

Interference of West Nile Virus with the Growth of Influenza A Virus

The observations on interference between a neurotropic and a pneumotropic virus were extended by the use of the West Nile virus. This virus differs from the yellow fever virus in being infectious for mice by the nasal route; large amounts, however, are required to kill by this route (23). Death results from invasion of the central nervous system, and not from pneumonia; and although pulmonary lesions are produced, they are usually small and differ in appearance from those caused by the influenza virus. The results of two experiments on reciprocal interference between the West Nile and the influenza viruses are given in Table VI.

In Experiment 1 cultures inoculated with influenza virus were reinoculated 24 hours later with graded doses of West Nile virus on the assumption that, in view of the results obtained with the yellow fever virus, interference might be difficult to detect if too much virus were used. After 48 hours' additional incubation, the cultures were titrated in young adult mice by the intranasal and intracerebral routes.

It will be observed in Table VI that while both viruses on intranasal inoculation killed mice or produced lesions in the lungs, the pathogenicity of the West Nile virus by this route was somewhat less than that of the influenza virus, and in addition, as shown by the correspondence of the LD_{50} and ID_{50} titers, pulmonary involvement due to the West Nile virus was found only in the mice which died and was not observed in animals which survived and were killed 10 days after inoculation. The intranasal LD_{50} and ID_{50} titers of the dually inoculated cultures indicate that infectivity by this route was due mainly to the influenza virus; but the intracerebral LD_{50} titers indicate that West Nile virus was not only present but had multiplied to about the same extent in those cultures inoculated with the three largest doses of West Nile virus as it had in the controls (*cf.* cultures 5a to 7a and 9a to 11a). However, comparison of the West Nile virus content of doubly infected culture 8a with that of West Nile control culture 12a, both of which had been inoculated with the smallest amount of this virus, shows that the latter culture contained 30 times more virus than the former. This is suggestive of the possibility that influenza virus can interfere with the growth of the West Nile virus if too large amounts of the latter are not used, but additional evidence is required before any unequivocal statement is warranted.

In Experiment 2 the effect of the West Nile virus on the propagation of influenza virus was investigated; the results are given in Table VI. It is at once

apparent from the low intranasal LD_{50} and ID_{50} titers of the doubly inoculated cultures, as compared with the titers of the influenza virus control cultures, that some interference with the growth of the influenza virus had occurred. The almost exact correspondence of the intranasal titers of the West Nile–influenza cultures and the West Nile control cultures would indicate that infec-

TABLE VI

Experiments on Reciprocal Interference between West Nile and Influenza A Viruses

Experiment No.	Culture flask	Inoculation of cultures: First inoculum* Virus	First inoculum* LD_{50}	Second inoculum* (24 hrs. after first inoculation) Virus	Second inoculum* LD_{50}	Virus titer of cultures (48 hrs. after second inoculation): Mice inoculated by (Intranasal route) LD_{50} titer	mean	ID_{50} titer	mean	(Intracerebral route) LD_{50} titer	mean
1	1a	PR8	$10^{5.2}$	None		$10^{-2.70}$	$10^{-2.85}$	$10^{-4.05}$	$10^{-4.25}$	0	
	2a					$10^{-2.85}$		$10^{-4.20}$		0	
	3a					$10^{-2.85}$		$10^{-4.30}$		0	
	4a					$10^{-3.00}$		$10^{-4.65}$		0	
	5a	PR8	$10^{5.2}$	W. Nile	$10^{5.1}$	$10^{-2.00}$	$10^{-2.40}$	$10^{-3.00}$	$10^{-3.35}$	$10^{-3.00}$	$10^{-2.75}$
	6a				$10^{4.1}$	$10^{-2.10}$		$10^{-3.30}$		$10^{-3.00}$	
	7a				$10^{3.1}$	$10^{-2.85}$		$10^{-2.95}$		$10^{-3.00}$	
	8a				$10^{3.1}$	$10^{-2.25}$		$10^{-3.10}$		$10^{-2.00}$	
	9a	None		W. Nile	$10^{5.1}$	$10^{-1.65}$	$10^{-1.65}$	$10^{-1.65}$	$10^{-1.65}$	$10^{-2.25}$	$10^{-2.50}$
	10a				$10^{4.1}$	$10^{-1.70}$		$10^{-1.70}$		$10^{-2.60}$	
	11a				$10^{3.1}$	$10^{-1.85}$		$10^{-1.85}$		$10^{-2.75}$	
	12a				$10^{2.1}$	$10^{-1.35}$		$10^{-1.35}$		$10^{-2.45}$	
2	1b	W. Nile	$10^{5.2}$	None		$10^{-1.10}$	$10^{-1.30}$	$10^{-1.10}$	$10^{-1.30}$	$10^{-2.60}$	$10^{-2.50}$
	2b					$10^{-1.30}$		$10^{-1.30}$		$10^{-2.75}$	
	3b					$10^{-1.50}$		$10^{-1.50}$		$10^{-2.20}$	
	4b	W. Nile	$10^{5.2}$	PR8	$10^{3.9}$	$10^{-1.40}$	$10^{-1.45}$	$10^{-1.50}$	$10^{-1.50}$	$10^{-3.00}$	$10^{-3.10}$
	5b					$10^{-1.45}$		$10^{-1.50}$		$10^{-3.20}$	
	6b					$10^{-1.50}$		$10^{-1.55}$		$10^{-3.10}$	
	7b	None		PR8	$10^{3.9}$	$10^{-2.05}$	$10^{-2.35}$	$10^{-4.10}$	$10^{-2.95}$	0	
	8b					$10^{-2.60}$		$10^{-3.80}$		0	
	9b					$10^{-2.39}$		$10^{-4.20}$		0	

* Viruses used were 24th passage PR8 and 40th passage West Nile in Experiment 1, and 35th passage PR8 and 47th passage West Nile in Experiment 2.

tivity of the dually inoculated cultures presumably was due to the West Nile component. However, some of the mice which died, or were killed, following inoculation of the West Nile–influenza cultures showed minimal lung lesions typical of those produced by influenza virus. If these were due wholly or in part to residual virus surviving from the inoculum seeded into the cultures, then complete or almost complete suppression of growth of the influenza virus

must have occurred. In order to check the validity of these results and to avoid misinterpretations which might arise from the ability of both viruses to produce lesions and death on intranasal inoculation, the experiment was repeated, using chick embryos as indicator animals for the presence of influenza virus.

Two experiments, identical except for the dosage and passage level of the viruses used, were done. Six flasks of culture medium were prepared, and four were inoculated with West Nile virus. After 24 hours' incubation, two of the West Nile cultures and the two non-inoculated cultures were inoculated with influenza virus. The cultures were incubated for an additional 48 hours and then centrifuged; the supernatants were used to prepare a series of fourfold dilutions in Tyrode's solution, which were inoculated in 0.1 ml. amounts into chick embryos by the allantoic route (34). Four embryos, 11 days old, were used for each virus dilution. The inoculated eggs were incubated at 37° for 48 hours and then chilled for 15 hours at 4° to facilitate harvesting of the allantoic fluids, which were tested for the presence of chick red blood cell agglutinins by the method of Hirst (35).

Dilutions of supernatant fluids from cultures infected with both viruses, or with West Nile virus alone, were also inoculated intracerebrally into mice in order to ascertain the West Nile virus content of the cultures.

Details of the experiments, and the results, are presented in Table VII.

Table VII shows that inoculation of chick embryos with relatively large amounts of West Nile culture virus did not result in the appearance in the allantoic fluid of agglutinins for chicken erythrocytes, whereas inoculation of embryos even with high dilutions of influenza culture virus did so. The presence of hemagglutinins in eggs inoculated with material from the dually infected cultures must therefore be ascribed to the action of influenza virus, and since only the lower dilutions of these cultures evoked the formation of hemagglutinins, interference with the multiplication of the influenza virus must have occurred. Comparison of the ID_{50} titers shows that in Experiment 2 the final concentration of influenza virus present in the dually inoculated cultures was only 0.1 per cent of that present in the corresponding control cultures, and that in Experiment 1 interference was even more marked, since the dually inoculated cultures contained only 0.001 per cent of the concentration of influenza virus present in the controls.

Search for Antiviral Substances in Tissue Cultures

Although interference appears to be based upon some subtle interaction between virus and living tissue, there is no evidence that it is effected through specific or non-specific antiviral substances (18, 20). Since a tissue culture represents a complete entity which can be readily examined in its entirety for the presence of such substances, it appeared worthwhile to see whether virus-inactivating agents could be detected in media used for cultivation of the interfering virus. Three experiments were done.

Experiment 1.—Each of one hundred and twenty flasks of culture medium was inoculated with $10^{3.15}$ LD_{50} of 695th culture passage 17DD High strain yellow fever virus and incubated

at 37°C. for 24 hours. At the end of this time obviously contaminated cultures were discarded and the remainder were pooled and transferred to 250 ml. centrifuge bottles. After horizontal centrifugation for 10 minutes at 1,000 R.P.M., most of the supernatant fluid was drawn off; a small aliquot was set aside for intracerebral titration in mice, and the rest was filtered through a Seitz EK pad and placed in the refrigerator overnight. This preparation was designated "fluid component;" its LD_{50} titer (prior to filtration) of 17DD virus was found to be $10^{-4.70}$. The sedimented tissue was transferred to graduated, conical bottomed 15 ml. tubes and centrifuged horizontally at 1,500 R.P.M. for 15 minutes. The supernatant fluid was drained off and the tissue was triturated with alundum to make a 20 per cent suspension, by volume, in 10 per

TABLE VII

Interference of West Nile Virus with the Growth of Influenza A Virus

Experiment No.	Inoculation of cultures: First inoculum* Virus	First inoculum* LD_{50}	Second inoculum* (24 hrs. after first inoculation) Virus	Second inoculum* LD_{50}	Culture flask	Virus titer of cultures (48 hrs. after second inoculation): Influenza virus — Hemagglutination ratio‡ of eggs inoculated with tissue culture dilution: Und.	1:4¹	1:4²	1:4³	1:4⁴	1:4⁵	1:4⁶	1:4⁷	1:4⁸	1:4⁹	1:4¹⁰	1:4¹¹	1:4¹²	Influenza virus: ID_{50} titer in eggs	West Nile virus: Intracerebral LD_{50} titer in mice
1	W. Nile	$10^{5.2}$	None		1a	0/4	0/4												<Und.	$10^{-3.60}$
					2a	0/4	0/4												<Und.	$10^{-3.20}$
	W. Nile	$10^{5.2}$	PR8	$10^{5.1}$	3a	2/4	2/4	1/4	1/4	1/4	0/4	0/4							$10^{-0.70}$	$10^{-3.10}$
					4a	4/4	3/4	0/4	1/4	0/4	0/4	0/4							$10^{-0.90}$	$10^{-3.15}$
	None		PR8	$10^{5.1}$	5a						4/4	4/4	4/4	3/4	4/4	3/4	0/4		$10^{-6.10}$	—
					6a						4/4	4/4	4/4	4/4	4/4	2/4	0/4		$10^{-6.00}$	—
2	W. Nile	$10^{5.3}$	None		1b	0/4													<Und.	$10^{-3.80}$
					2b	0/4													<Und.	$10^{-4.00}$
	W. Nile	$10^{5.3}$	PR8	$10^{5.2}$	3b	3/4	4/4	3/4	3/4	2/4	1/4	0/4							$10^{-2.15}$	$10^{-2.50}$
					4b	3/4	4/4	4/4	4/4	2/4	0/4	0/4							$10^{-2.25}$	$10^{-2.75}$
	None		PR8	$10^{5.2}$	5b								4/4	2/4	0/4	2/4	1/4	0/4	$10^{-5.15}$	—
					6b								4/4	1/4	1/4	2/4	1/4	0/4	$10^{-5.15}$	—

* Viruses used were 58th passage West Nile and 6th passage PR8 in Experiment 1, and 62nd passage West Nile and 13th passage PR8 in Experiment 2.

‡ The numerator represents the number of allantoic fluids producing hemagglutination, the denominator the number of eggs inoculated.

cent normal human serum–Tyrode's solution. This suspension was centrifuged horizontally at 3,500 R.P.M. for 40 minutes and the supernatant liquid, designated "tissue extract," was drawn off; a portion was titrated intracerebrally in mice for virus content and the rest was passed through a Seitz EK filter and stored in the refrigerator overnight. The LD_{50} titer of the unfiltered extract was $10^{-7.80}$

The following day the fluid component and the tissue extract were both diluted with an equal volume of serum–Tyrode's solution and divided into two portions, one of which was heated in a water bath for 3 hours at 56°C. and the other filtered through collodion membranes with an average pore diamater of 12 mμ. Each of the four preparations was tested for the

presence of virus by inoculating a portion intracerebrally into a group of 12 mice. The animals were examined daily for 3 weeks, and the brains of mice found dead were passaged to exclude the possibility that death resulted from infection with 17DD virus. The results indicated that none of the four preparations contained demonstrable active virus; each preparation was, therefore, used for the cultivation of the Asibi and West Nile viruses, as follows:—

Each preparation was distributed into culture flasks in 4.0 ml. amounts and minced chick embryo was added. Controls consisted of the usual chick embryo and serum-Tyrode mixture. Sets of flasks of each test medium and control medium were then inoculated with $10^{5.3}$ LD_{50} of 80th culture passage Asibi or with $10^{5.2}$ LD_{50} of 49th culture passage West Nile virus. As additional controls, flasks of each of the test media were left uninoculated on the possibility that active 17DD virus might be present, despite failure to detect it by mouse inoculation, and would multiply sufficiently to attain a concentration infectious for mice.

All cultures were incubated at 37°C. for 72 hours, and the supernatant fluids from each set of cultures inoculated with virus were pooled according to the test medium and virus and titrated intracerebrally in mice. Undiluted supernatants from the uninoculated cultures were injected intracerebrally into mice, 12 animals per preparation; all survived, indicating that the test media were free of active 17DD virus.

The results of the experiment are shown in Table VIII.

Experiment 2.—This experiment was similar to the preceding one. Sixty flasks of culture medium were inoculated with $10^{2.75}$ LD_{50} of 702nd passage 17DD High strain virus, and treated as before. The LD_{50} titers of the fluid component and the tissue extract were $10^{-4.60}$ and $10^{-8.40}$ prior to Seitz filtration. The ultrafiltrates and the heated preparations contained no demonstrable active virus. An aliquot of each preparation was tested for neutralizing antibodies to yellow fever virus and the rest was used for the cultivation of West Nile virus. Test and control media were prepared as before and inoculated with $10^{3.0}$ LD_{50} of 51st passage West Nile virus; their virus content was determined after 72 hours' incubation.

Table VIII gives the results of this experiment.

Experiment 3.—One hundred and eighty flasks of culture medium were prepared, and half of them were inoculated with $10^{3.2}$ LD_{50} of 715th passage 17DD High strain virus. The entire set of cultures was incubated at 37° for 24 hours, then made into two large pools of infected and non-infected material. The fluid component and tissue extract derived from each pool were subjected to ultrafiltration only; none of the material was heated. The infected fluid component and tissue extract prior to filtration had LD_{50} titers of $10^{-5.50}$ and $10^{-6.40}$ respectively; after filtration, neither was infectious for mice. The ultrafiltrates of the fluid components and tissue extracts were used to prepare cultures which were inoculated with $10^{3.2}$ LD_{50} of 69th passage West Nile virus or with $10^{2.8}$ LD_{50} of 73rd passage Venezuelan equine encephalomyelitis virus. The cultures were incubated for 72 hours at 37° and titrated for virus content. The results are shown in Table VIII.

Table VIII gives the results of the three experiments. Experiments 1 and 2 show that the Asibi and West Nile viruses grew as well in cultures in which the liquid portion consisted of heated tissue extract or fluid component from cultures previously infected with the 17DD virus as they did in the control medium. As regards the ultrafiltrates of infected cultures, the Asibi virus grew as well in cultures made with ultrafiltered tissue extract as in the controls. The West Nile virus, on the other hand, in one experiment multiplied equally well in media

containing ultrafiltered tissue extract and in control media, but in the other attained a lower titer in the test medium than in the control; the difference, roughly tenfold, is of dubious significance, since the titrations were based on the use of tenfold dilutions. Where ultrafiltered fluid component was used as culture medium, there appeared to be a deleterious effect on the propagation of both the Asibi and West Nile viruses. In the case of the Asibi virus the difference in virus content of the test and control media was about a hundredfold, while the difference in West Nile virus content of similar media was roughly tenfold in each of the two experiments. These results are not susceptible of unequivocal explanation, since tissue extracts which *a priori* should be richer in inhibitor substances, with a single exception (West Nile virus, Experiment 1) exerted no such deleterious action on the growth of the second virus; in addition,

TABLE VIII

Examination of Fluid and Cellular Components from Tissue Cultures Infected with 17DD High Strain Yellow Fever Virus for Presence of Non-Specific Antiviral Substances

Experiment No	Culture inoculum		LD_{50} titer of virus in cultures whose fluid portion was derived from						LD_{50} titer of virus in control medium
			Cultures previously infected with 17DD virus				Non-infected cultures		
			Tissue extract		Fluid component		Tissue extract	Fluid component	
	Virus*	LD_{50}	Ultrafiltered	Heated	Ultrafiltered	Heated	Ultrafiltered	Ultrafiltered	
1	Asibi	$10^{5.3}$	$10^{-4.55}$	$10^{-4.30}$	$10^{-2.10}$	$10^{-4.40}$			$10^{-4.30}$
	W. Nile	$10^{5.3}$	$10^{-2.40}$	$10^{-3.70}$	$10^{-2.15}$	$10^{-2.30}$			$10^{-2.50}$
2	W. Nile	$10^{3.0}$	$10^{-2.25}$	$10^{-2.45}$	$10^{-2.15}$	$10^{-2.95}$			$10^{-2.30}$
3	W. Nile	$10^{3.2}$	$10^{-4.50}$		$10^{-4.40}$		$10^{-4.25}$	$10^{-4.40}$	$10^{-5.00}$
	Ven. e. e.	$10^{3.8}$	$10^{-5.80}$		$10^{-6.25}$		$10^{-6.25}$	$10^{-5.80}$	$10^{-6.25}$

* Viruses used were 80th passage Asibi; 49th, 51st, and 63rd passage West Nile; and 73rd passage Venezuelan equine encephalomyelitis.

in Experiment 3, neither ultrafiltered tissue extracts nor fluid components from 17DD-infected cultures contained anything inhibitory to the growth of either the West Nile or Venezuelan equine encephalomyelitis virus. Since the virus content of four of the five series of cultures containing ultrafiltered fluid component was practically identical with that of the controls, or differed from them by differences within the range of experimental error of the titration method, it would appear that interference in tissue cultures is not attributable to non-specific substances arising as a result of multiplication of the initially inoculated virus. Additional information on this point, however, is desirable.

Humoral immunity does not seem to be involved in the interference phenomenon, as none of the four types of culture preparations tested contained antibodies to the yellow fever virus, although the highly sensitive extraneural neutralization test in young mice (31) was used.

Experiments on Interference in Mice and Chick Embryos

Several experiments were done to determine whether interference could be produced in mice and chick embryos by using the method employed in the tissue culture experiments.

Mice.—Three-day-old mice were inoculated subcutaneously with 0.03 ml. of undiluted 17DD High strain yellow fever virus, tissue culture supernate. Twenty-four hours later the mice were reinoculated subcutaneously with 0.03 ml. of dilutions of tissue culture Venezuelan equine encephalomyelitis or West Nile virus, or with dilutions of monkey serum Asibi yellow fever virus; Asibi serum virus was used rather than tissue culture virus because of its higher infectivity by the extraneural route. Groups of 6 mice were inoculated with each virus dilution, and the viruses were titrated by parallel inoculation into normal 4-day-old mice, 6 animals per dilution.

TABLE IX

Attempts to Produce Interference in 3-Day-Old Mice by Subcutaneous Inoculation of Viruses

Experiment No.	Inoculation of mice			LD$_{50}$ titer of second virus in test and in control mice
	First inoculum*		Second inoculum* (24 hrs. after first inoculation)	
	Virus	LD$_{50}$‡	Virus	
1	17DD	$10^{8.2}$	Ven. e. e.	$10^{-5.25}$
	None		Ven. e. e.	$10^{-5.55}$
2	17DD	$10^{8.0}$	West Nile	$10^{-2.50}$
	None		West Nile	$10^{-3.45}$
3	17DD	$10^{2.9}$	Asibi	$10^{-5.70}$
	None		Asibi	$10^{-7.85}$

* In Experiment 1, 659th culture passage 17DD virus and 19th culture passage Venezuelan equine encephalomyelitis virus were used.
In Experiment 2, 661st culture passage 17DD and 17th culture passage West Nile virus were used.
In Experiment 3, 689th culture passage 17DD and 42nd *rhesus* passage Asibi serum virus were used.
‡ Intracerebral LD$_{50}$.

The results of the experiments with mice are summarized in Table IX.

Comparison of the LD$_{50}$ titers in Table IX shows that the Venezuelan equine encephalomyelitis and West Nile viruses multiplied as readily in mice previously inoculated with 17DD yellow fever virus as in untreated controls. Some interference with multiplication of Asibi virus occurred, however, since mice inoculated with 17DD virus and subsequently with small amounts (high dilutions) of Asibi virus survived, so that the resultant LD$_{50}$ titer of Asibi virus in the test mice was $10^{-5.70}$ as compared with $10^{-7.85}$ in the controls, a 140-fold difference.

Chick Embryos.—In the first experiment, 9-day-old eggs were used; windows were cut into the shells and the chorioallantoic membranes were dropped. Part of the eggs were inoculated on the membrane with 0.03 ml. of a freshly prepared suspension of 17D-EP Low strain (egg

passage (22)) yellow fever virus, obtained by passing infected chick embryos through a colloid mill, centrifuging the resulting pulp, and diluting the supernatant juice tenfold with serum-saline. The remainder were similarly inoculated with the supernatant fluid from a centrifuged 10 per cent saline suspension of normal chick embryos triturated in a blendor. The eggs were incubated at 37° for 24 hours, candled, divided into groups of 6 or 7, and reinoculated on the membrane with 0.03 ml. of dilutions of Venezuelan equine encephalomyelitis egg passage virus. Six eggs inoculated with 17D-EP were reinoculated with the 10 per cent normal chick embryo suspension and reserved as controls on the multiplication of the 17D virus. After 5 days' incubation the embryos were triturated with serum-saline in a blendor, and serial tenfold dilutions made from the centrifuged supernatant fluid were inoculated intracerebrally into mice.

Eggs inoculated with the Venezuelan equine encephalomyelitis virus were candled daily, and the mortality was recorded during a 10 day observation period (36); the number of embryos which died within this period was used to compute the LD_{50} titers of the virus.

TABLE X

Effect of 17D Strain of Yellow Fever Virus on Growth of Venezuelan Equine Encephalomyelitis Virus in the Chick Embryo

Experiment No.	Initial inoculum*		Second inoculum*									
	Virus	LD_{50}‡	Virus	Mortality ratio of chick embryos inoculated with virus dilution								LD_{50} titer of virus
				10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	
1	17D	$10^{3.8}$	Ven. e. e.	6/6	6/7	7/7	3/7	5/7	0/7			$10^{-7.85}$
	None		Ven. e. e.				6/6	5/6	4/6	3/6	0/6	$10^{-9.55}$
2	17D	$10^{3.2}$	Ven. e. e.	7/7	7/7	6/7	4/7	3/7	4/7	0/7		$10^{-7.90}$
	None		Ven. e. e.				7/7	5/7	3/7	1/7	1/7	$10^{-8.35}$

* In Experiment 1, 43rd egg passage 17D-EP virus and 59th egg passage Venezuelan equine encephalomyelitis virus were used.

In Experiment 2, 41st egg passage 17D-EP virus and 59th egg passage Venezuelan equine encephalomyelitis virus were used.

‡ Mouse intracerebral LD_{50}.

The second experiment differed from the first only in that the eggs were inoculated with 17D-EP virus or with normal chick embryo suspension on each of 2 successive days before being inoculated with the Venezuelan equine encephalomyelitis virus.

The results of the experiments are presented in Table X.

Intracerebral titration in mice of embryos inoculated with 17D virus alone gave LD_{50} titers of $10^{-4.50}$ in Experiments 1 and 2 respectively, showing that multiplication of this virus had occurred. Comparison of the LD_{50} titers given in Table X for Venezuelan equine encephalomyelitis virus cultured in 17D-infected and in non-infected eggs shows that this virus attained a fiftyfold greater titer in the non-infected eggs than in those previously infected with 17D virus. The difference was considered on the border-line of significance and was interpreted as indicating a possible interfering effect of 17D virus on the

Venezuelan equine virus. In order to enhance, if possible, the degree of interference, Experiment 2 was done, in which eggs were inoculated with 17D virus on 2 successive days prior to inoculation with the Venezuelan virus. This procedure, however, had no effect, as is shown in Table X. The Venezuelan equine virus multiplied to about the same extent in both the 17D-infected and in the control eggs; the difference between the LD_{50} titers was only ninefold, and within the limits of experimental error of the titration method. In view of these results, the suggestion of an interfering effect in Experiment 1 is probably more apparent than real.

The experiments described in this section thus indicate that on the whole the experimental conditions determining the occurrence of interference in tissue cultures are different from those required when a host such as the mouse or chick embryo is used, and presumably must be ascertained for each host species.

DISCUSSION

Although Andrewes (20) has shown that interference between two strains of influenza A virus can occur in tissue cultures, the possibilities of this method have not been fully explored. Inasmuch as the simplicity of tissue culture media offers, in some respects, definite advantages over animal hosts, the feasibility of using this approach to the study of interference was investigated.

Interference in cultures was observed to occur between a number of viruses used in several combinations, so that the method appears applicable to any virus capable of satisfactory multiplication *in vitro*. The chief difficulty resides in obtaining for certain systems an adequate means for determining whether or not interference has taken place. In the present work, when two viruses possessed different tissue tropisms, *e.g.*, influenza and yellow fever viruses, interference with the growth of one or the other was readily detected by mouse inoculation. When the tissue affinities of both viruses were the same, other means of distinguishing between them had to be resorted to. In the case of the influenza virus and of the West Nile virus, which has a limited capacity to produce lung lesions, distinction in the mouse rested on the differences in the type of pulmonary lesion and symptoms of infection produced, and in the chick embryo on the ability of influenza virus to produce agglutinins for chicken erythrocytes, while the neurotropic viruses were distinguished by differences in infectivity by extraneural routes for mice of selected known ages (29). Unfortunately, differentiation by extraneural infectivity was possible in only one direction, and since no simple, satisfactory alternative method was available, reciprocal interference could not be investigated.

Multiplication of the 17DD High strain of yellow fever virus resulted in suppression of growth of the Asibi strain of the same virus added later; since these viruses are not only antigenically related, but 17DD is also a derivative of the Asibi strain, interference might be expected. The finding is of interest,

however, in showing that interference between strains of yellow fever virus occurs in cultures as well as in animals and, taken in conjunction with recent similar findings for influenza virus, indicates that there may be a parallelism between interference of two viruses *in vivo* and *in vitro*.

An interfering effect was also observed between viruses which are antigenically distinct although possessing, in the mouse, the same tissue tropism. The growth of both the West Nile virus and Venezuelan equine encephalomyelitis virus could be suppressed when these were added to cultures infected 24 hours before with 17DD yellow fever virus. The suppressive action of 17DD cannot be ascribed to its high adaptation to tissue cultures, since low passage West Nile virus exerted as pronounced an effect on growth of the Venezuelan equine encephalomyelitis virus as did the 17DD virus.

Interference in tissue culture may also occur between viruses which differ not only in their antigenic constitution but also in their tissue tropism. It is recognized, however, that the difference in tropism may be more apparent than real, since *in vitro* conditions may conceivably be such that both viruses utilize the same tissue cells. Nevertheless, cultures infected with either West Nile virus or 17DD yellow fever virus suppressed the growth of influenza virus added later; under the same experimental conditions, however, interference in the reverse direction was not demonstrable.

The diverse nature of the viruses between which interference has been demonstrated indicates that a fundamental mechanism common to all the systems studied is responsible for the phenomenon. What the nature of this mechanism may be can only be speculated on at present. It apparently is not associated with antibodies, not only because antibody formation has not been detected, but also because interference can occur between viruses totally unrelated immunologically, nor do non-specific antiviral substances appear to be involved since the fluid and tissue extracts from infected cultures can support the growth of another virus when fresh cells are added.

The mechanism is, however, indubitably associated with some interaction of virus and susceptible cells of the tissue culture. Whatever its nature, the reaction is consummated with such rapidity that within a matter of hours the culture tissues become resistant to superinfection by a second virus, to which they are normally susceptible. Resistance to superinfection is only relative, however, and may be partially broken down when excessive amounts of the second virus are added. This is exemplified by the ability of 17DD yellow fever virus and the West Nile virus to suppress completely the growth of the Venezuelan equine encephalomyelitis virus, when small quantities of the latter were used. When large amounts of the Venezuelan virus were used, dual infection of the cultures occurred, but some interference with the growth of the Venezuelan virus took place. On the other hand, the influenza virus did not interfere to any obvious extent with the multiplication of West Nile virus or 17DD

yellow fever virus, even though relatively small quantities of these were used; the results with yellow fever virus indicated, rather, that this virus may have adversely affected the multiplication of the influenza virus despite its advantage of a 24 hour start in growth. The most obvious interpretation appears to be that in both cases the outcome represents the resultant of the differences in multiplication rates of the viruses concerned and of the quantity of virus in the inocula; this does not, however, adequately explain why in one case the multiplication of virus in the second inoculum is adversely affected by that of virus in the initial inoculum, and in another the opposite effect is produced (19).

While the available evidence points to some interaction between virus and susceptible cell as the basis for the interference phenomenon, there are as yet insufficient data for more than conjecture as to whether the reaction takes place on the surface of the cell or within the cell. Tissue cultures, representing a relatively simple *in vitro* entity, afford a means for detailed analysis of the entire reacting system, and may provide a useful technique for the study of the mechanism of interference.

SUMMARY

The influence of one virus on the growth of another in tissue culture was investigated.

The 17DD High strain of yellow fever virus was found capable of completely suppressing the growth of both the Asibi strain of the same virus and of the heterologous West Nile virus, even when these were added to the cultures in large amounts.

The 17DD High strain of yellow fever virus and the West Nile virus produced either partial or complete suppression of growth of the Venezuelan equine encephalomyelitis virus, depending upon the quantity of the latter inoculated into the cultures. Owing to lack of methods for the detection of interference except in a single direction, reciprocal interference with these viruses could not be investigated.

The 17DD High strain of yellow fever virus and the West Nile virus were able to suppress completely, or almost completely, the growth of influenza A virus added to the infected cultures in maximal amounts. Interference in the reverse direction, even with the use of small amounts of the neurotropic viruses, was not demonstrable.

Cultures infected with the 17DD High strain of yellow fever virus were examined for the presence of neutralizing antibodies and non-specific antiviral substances; neither was found present.

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