TABLE VI.
Comparison of Results of Antigenic Extinction Titration of N.I.H. Control Vaccine in Young and Old Mice.

Dilutions of vaccine inoculated	Challenge Virus*					
	PR8 F198 M720 E2		PR8 F198 M593 E64		Weiss F3 M32 E58	
	Serum from		Serum from		Serum from	
	Old mice	Young mice	Old mice	Young mice	Old mice	Young mice
10	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0 0
20	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	+++ +
40	0 0 0 0	0 0 0 0	0 0 0 0	0 0+ +	0 0 0 0	0+ + +
80	0 0 0 0	0 0 0 0	0 0+ +	+ + + +	+ + + +	+ + + +
160	0 0 0 0	0 0 0 0	0 0 0 0	+ + + +	0+ + +	+ + + +
320	0 0 0 0	0 0 0 0	0 0 0+	+ + + +	+ + + +	+ + + +
640	0 0 0 0	0 0 0 0	0 0+ +	0+ + +	+ + + +	+ + + +
1280	0 0 0 0	0+ + +	+ + + +	+ + + +	+ + + +	+ + + +
2560	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
5120	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Controls	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Symbols same as Table I.
* Approximately 1000 50% egg-infecting doses of each strain were used.

The use of chick embryos rather than mice as the indicator medium for virus neutralization offers the advantages of completion in 2 days rather than 10 days, applicability to egg-adapted strains and sharpness of results, as well as being less costly.

The importance of maintaining the identity of virus in vaccine and virus used as test antigen for measuring the antibody response induced by immunization has been illustrated and discussed.

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Experimental Infection of Domestic Animals with Japanese B Encephalitis Virus.*

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During the summer of 1946 a series of experiments were carried out on Okinawa with the general purpose of investigating the part that domestic animals might play in the epidemiology of Japanese B encephalitis.[1] Workers from Naval Medical Research Unit Number 2 had presented evidence that horses might have such a role.[2] It appeared desirable to obtain additional evidence on this point and also to study the other domestic animals which were common on the island during the epidemic of 1945. The experiments were designed (1) to determine how long virus persisted in the blood of experimentally infected animals, (2) to determine whether the virus was pathogenic for any of the species studied and (3) to study the time of appearance and increase of neutralizing and complement-fix-

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1 Thomas, L., and Peck, J. L., U. S. Naval Medical Research Unit No. 2, Official Report, 1945, unpublished.

2 Hodes, H. L., Thomas, L., and Peck, J. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 220.

ing antibodies.

Materials and Methods. Laboratory Facilities and Animals. A field laboratory was set up in a quonset hut of the former Military Government Hospital at Goya. An adequately screened adjoining building housed the smaller animals, while horses were kept in an especially constructed stable designed by previous workers.[2] Swiss albino mice 1 to 3 months of age were used for passage and isolation of virus. These animals were in good condition on arrival, but suffered, to some extent, from intercurrent infections during the period of the experiments.

Virus Strain. The Okinawan strain of Japanese encephalitis, isolated by Thomas and Peck,[3] was used in all experiments. This strain had been through from 13 to 15 mouse brain passages. Brains were stored, during early experiments, in the freezing compartment of an icebox and later in an icebox at a temperature between 4 and 10°C.

Route of Infection and Dose of Virus. All animals were infected by the intravenous route with 1.0 ml of a 10^{-2} dilution of a fresh mouse brain suspension. 10% normal rabbit serum saline was used as diluent. Concurrent titrations were not done, but subsequent intracerebral titrations in mice suggested that the titer of these brains was of the order of 10^{-7}.

Experimental Animals. Pigs, goats, and chickens were obtained from the south central part of Okinawa. The pigs were estimated to be 4 months old, the goats 4 to 8 months, and the chickens less than a year old. Domestic Muscovy ducks were obtained from the village of Hentona and were at least 2 years old. Young horses could not be found on the main island and, for this reason, were procured on the small island of Iheya Shima off the northern tip of Okinawa where much of the horse-breeding has been carried on in recent years. Three were colts 2 to 4 months of age, and the fourth was a 2-year-old.

Recovery of Virus from Blood. Animals were bled before inoculation and 1, 3, 6, 9, and 12 days after inoculation. After the blood had coagulated, 0.03 ml of the serum with a small quantity of suspended cells was injected intracerebrally into each of 3 or 4 mice. Deaths occurring between the 4th and 10th days with characteristic signs and absence of other apparent illness were considered to be probably due to the encephalitis virus. However, in order to better establish this point, brains of moribund or recently dead mice were harvested, stored, and later transported to Berkeley for definite identification by passage and neutralization with specific antiserum. Virus was still present in many of the brains after 6 weeks of storage at ordinary icebox temperatures, but may have been lost from others.

Neutralization Tests. Serial 10-fold dilutions of infected mouse brain suspensions in 10% normal rabbit serum broth were mixed with equal amounts of undiluted, noninactivated, test serum. After incubation for 2 hours at room temperature, the serum-virus mixture was inoculated intracerebrally in 0.03 ml amounts into groups of mice 2 to 6 weeks old. The LD_{50} of the virus-suspension was determined by titration under similar conditions with normal rabbit serum and the neutralizing capacity of the test serum expressed in terms of a neutralization index. Test mice were observed for 14 days.

Complement-fixation Tests. Mouse brain antigens were prepared by the method of Casals and Palacios.[4] Sera were inactivated for 30 minutes at 60° or at 65°C, if necessary. At least 2 other mouse brain antigens were run concurrently with each serum. Overnight fixation at 4°C was used. Results were expressed in terms of the original serum dilution.

Results. Recovery of Virus from Blood. The results of experiments with each species are summarized in Table I. Virus was recovered from all the pigs and ducks 24 hours after inoculation, in sufficient amounts to kill all tested mice within 7 days. Smaller amounts appeared to be present in the blood

[3] Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

[4] Hammon, W. McD., Reeves, W. C., and Burroughs, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 304.

TABLE I.
Results of Intracerebral Inoculation of Mice with the Blood of Experimental Animals.

Species	No.	Pre-inoc.	Days after inoculation 1	3	6	9	12
Horse	1	2/3*	0/4	0/3	0/3	0/3	0/3
	2	1/3	0/4	0/3	0/3	0/3	0/3
	3	1/3	0/4	0/3	0/3	0/3	0/3
	4	0/3	1/4	0/3	0/3	0/3	0/3
Goat	1	0/2	1/3	1/3	0/4	0/4	0/4
	2	0/2	0/3	0/3	0/4	0/4	0/4
	3	0/3	0/3	0/3	0/4	0/4	0/4
	4	0/3	0/3	0/3	0/4	0/4	0/4
Pig	1	0/2	3/3†	0/3	(dead)		
	2	2/2	3/3†	0/4	0/4	0/4	—
	3	0/2	3/3†	0/3	0/4	(dead)	
Duck	1	1/2	3/3†	1/4	0/4	1/4	—
	2	0/2	3/3†	0/4	1/4	1/4	—
	3	1/2	3/3†	0/4	0/4	1/3	—
	4	0/2	3/3†	0/4	0/4	0/3	—
Chicken	1	0/3	1/4	1/4	0/3	0/2	0/3
	2	1/3	1/4	3/4	0/3	0/2	0/3
	3	0/3	1/4	2/3	0/3	0/2	0/3
	4	0/3	3/4	2/4	0/3	1/2	0/3

* Dead/Total.
† Specificity of deaths confirmed by passage and/or neutralization.

of chicken No. 4 on the 1st and 3rd days and of chickens No. 2 and No. 3 on the 3rd day. Minimal amounts may have been recovered from horse No. 1 on the day following inoculation and from goat No. 1 on the 1st and 3rd days and in certain of the later duck bloods, but the specificity of these deaths was not established. It seems certain that, if virus was present, the amount was small.

Evidence of Illness. None of the goats or ducks appeared ill at any time. One of the chickens appeared listless 5 days after inoculation but soon recovered. The 4 horses likewise showed no rise in temperature or evidence of illness. The 3 young colts were subsequently shown to have high levels of antibody before inoculation, and presumably only the 2-year-old (horse No. 4) was nonimmune.

A very different picture was observed with the pigs. All 3 appeared ill on the 5th day, with ataxia, drowsiness, irritability, anorexia, and conjunctivitis the most notable findings. Temperatures ranged from 103[2] to 106. Pigs No. 1 and No. 3 became progressively worse and died on the 6th and 8th days, respectively. Pig. No. 2 made a gradual recovery, but remained ataxic and walked with a peculiar, high-stepping gait. Complete autopsies were done on both animals from 4 to 8 hours after death. There were no grossly abnormal findings other than diffuse hyperemia of the brain in each case and cerebellar necrosis in pig No. 1. The cisternal fluid of pig No. 1 contained more than 500 leucocytes. Suspensions of various parts of both brains were inoculated into mice, but virus was not recovered.

Neutralizing and Complement-fixing Antibodies. The results of serial determination of neutralizing antibody levels are presented in Table II, and those of complement-fixing antibody levels in Table III. The single susceptible horse showed definite evidence of neutralizing antibody on the 6th day, with a further rise on the 12th day. All 4 goats had antibody by the 6th day and failed to rise further during the following 18 days. Pigs No. 2 and No. 3 also had antibody on the 6th day, the day before the latter died. The response in the ducks was similar. Two of the 4 chickens neutralized strongly on the

TABLE II.
Serial Determinations of Intracerebral Neutralizing Antibody in Experimental Animals.

Species	No.	Pre-inoc.	Days after inoculation 1	3	6	9	12	24-28
Horse	1	+++*	+++	+++	+++	++	+++	
	2	+++		+++	+++	++	+++	
	3	++	+	+	+	+	++	
	4	0	0	±	±	+	+	
Goat	1	0	0	0	+	+	+	+
	2	0	0	±	+	+	+	+
	3	0	0	0	+	+	+	+
	4	0	0	0	+	+	+	±
Pig	1	0	0	±	(dead)			
	2	0	0	±	+	++		++
	3	0	0	0	+	(dead)		
Duck	1	0	0	0	+	0		++
	2	0	0	±	±			++
	3	0	0	0	+	+		++
	4	0		0	±	+		++
Chicken	1	0			0		±	
	2	0			++		+++	
	3	0			±		+	
	4	0			++		++	

* Graded according to neutralization index: 0 = 0 LD_{50}; ± = 10-32 LD_{50}; + = 32-100 LD_{50}; ++ = 100-320 LD_{50}; +++ = 320-1000 LD_{50}.

TABLE III.
Serial Determination of Complement-fixing Antibody Titers of Experimental Animals.

Species	No.	Pre-inoc.	Days after inoculation 1	3	6	9	12	28
Horse	1*	8	8	8		8	8	
	2*	32	8	32	64	32	16	
	3*	4	8	4	4	4	4	
	4	4	8	8	8	32	32	
Goat	1	8	16	16	8	16	16	16
	2	0	0	8 (?)	0	0	0	32
	3	0	0	4		8		16
	4	0	0			8	4	64
Pig	1	0	0	0 (Dead)				
	2	0	0					16
	3	4	0	0 (Dead)				

* Showed neutralizing antibody in pre-inoculation serum.

6th day, and on the 12th day these 2 had higher neutralization indices than any of the other species tested. The proportionately greater inoculum in these small birds may have contributed to this difference.

Complement-fixation tests were performed only with the sera of the mammals. Three animals which had shown no neutralizing antibody before inoculation showed fixation in their initial bleedings in dilutions of 1:4 and 1:8. This may represent specific antibody persisting after the disappearance of neutralizing antibody, for sera from the same species of animals bled in New Caledonia uniformly failed to show fixation even at a dilution of 1:4. In horse No. 4 the titer rose rapidly by the 9th day and had reached 64 on the 12th day, at which time the neutralization index was still relatively low. The titer of one goat began to rise on the 9th, of an-

other on the 12th day, while that of a third did not show a rise until the last bleeding. The fourth goat failed to show a significant rise. The single surviving pig had a titer of only 16 in his latest bleeding, 28 days after inoculation.

Discussion. When the data obtained in these experiments is considered along with the earlier horse experiment of Thomas and Peck[2] and the chicken experiments of Hammon *et al.*,[5] it appears that only one of the species so far tested, namely the goat, can reasonably be excluded from consideration as a possible source of infection for the insect vectors of this virus. There is no doubt that experiments of this sort leave much to be desired, since both the route and dosage of virus may differ greatly from those occurring in the natural infection. There obviously remains a broad field for investigation before this question can be settled.

The usefulness of the complement-fixation test, stressed by other workers,[4,3] is further emphasized by this animal data. Complement-fixing antibody appeared as early as neutralizing antibody and its titer then rose sharply, whereas the titer of the latter remained at relatively low levels during the period of observation. This relationship is very different from that seen in experimental infection of horses with Western equine encephalomyelitis virus, in which the neutralizing antibody appears earlier and rapidly reaches a high level.

The finding of antibody in colts 2 to 4 months of age is of some interest. These animals had been foaled during late winter or early spring seasons and were procured at a time before any clinical encephalitis had appeared. It seems, therefore, unlikely that they had acquired antibody as a result of infection. Furthermore, their dams, along with 6 other horses, had been bled on Iheya Shima, and all showed high levels of neutralizing antibody. The data point toward maternal transmission as the probable explanation of antibody in the colts.

The observation that all 3 inoculated swine developed signs of encephalitis and that 2 of the 3 died is of considerable interest. Despite the fact that virus was recovered from the blood of each animal on the day following injection, the failure to recover virus from the brains makes it impossible to conclude that the experimental agent was responsible for their illnesses or deaths. Intercurrent infection by some agent pathogenic for swine could not be excluded even though (a) the animals had been observed in isolation from other porcine contacts for more than 10 days before any showed signs of illness; (b) the clinical picture suggested an infection of the central nervous system and the autopsies showed no pathological findings outside the central nervous system; and (c) a fourth pig, which was kept in the same pen throughout the experiments but not inoculated with virus, showed no sign of illness. It is obvious, however, that the pathogenicity of this virus for swine can be established only by further experiments. The epidemiological and veterinary implications of this possibility appear to warrant investigation.

Summary. 1. Okinawan horses, goats, pigs, ducks, and chickens were inoculated intravenously with the virus of Japanese B encephalitis in order to obtain additional data regarding their role as natural reservoirs of disease.

2. Virus was recovered from the blood of pigs and ducks 24 hours after intravenous inoculation and was probably present for a longer period in chickens.

3. Two of 3 inoculated pigs died with characteristic signs of encephalitis while the third recovered after a long illness of a similar nature. Virus was not recovered from the brains of the animals which died. The other species showed no clinical evidence of infection.

4. Complement-fixing antibodies in experimental animals rapidly reached high levels, whereas neutralizing antibody levels rose more slowly.

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[5] Meiklejohn, G., and Dean, B., unpublished data.

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Chemical Antagonism of Pteroylglutamic Acid in a Pig; Hematopoietic Effect of Extrinsic and Intrinsic Factors.*

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It has been established that pteroylglutamic (folic) acid (PGA) is concerned with the formation of various cells of the blood, particularly erythrocytes and granulocytes. The vitamin has been studied in several animal species, including chicken, turkey, rat, dog, and monkey, as well as in man.

In man, with various types of macrocytic anemia, including that seen in pernicious anemia and in sprue, a striking hematologic response is usually obtained when PGA is administered.[1,2,3,4] This response cannot be differentiated clearly from that characteristically produced by refined liver extracts,† or from that caused by the administration, in pernicious anemia in relapse, of normal gastric juice (containing intrinsic factor) together with a heat-stable substance (extrinsic factor) found in beef skeletal muscle, casein, and other natural materials.[5] The roles of the non-PGA antipernicious anemia (APA) factor of liver extracts and of the extrinsic factor have been most difficult to study, because the effects produced in man have not been demonstrable in animals, even in those with anemia or leucopenia induced by a deficiency of PGA.[6,7,8]

Only swine have offered promise for studies of this character. The liver of this species is rich in the APA factor and is widely employed in the manufacture of liver extracts used in the treatment of macrocytic anemias. It was shown by Miller and Rhoads[9] that, under certain dietary conditions, swine develop an anemia that responds to injections of liver extract. Most striking, however, was the report of Cartwright, Wintrobe and Hum-

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† Refined liver extracts contain insignificant amounts of determinable PGA, often less than 0.001 mg per cc.

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