(100 mg/kg) and U/40 regular insulin (0.4 U/kg). There was no significant difference in the acid response to these two stimuli (U = 17, $p>0.05$).

Atropine sulfate (U.S.P., 1.5 mg/kg), injected simultaneously with 2-D.G. (100 mg/kg) in 6 experiments, abolished the gastric response.

Discussion. Our results indicate that the rat gastric acid response to 2-D.G. is "all or none" over the dose range of 50-400 mg/kg and that it is not larger than the response obtained with insulin. Hirschowitz and coworkers(1,2), however, have demonstrated a graded dose-response relationship in the range of 25-100 mg/kg when the drug is injected intravenously into conscious dogs; they also reported that the acid response to 2-D.G. in the dog and man exceeded that produced by insulin. Species difference and/or the difference in route of administration may account for the lack of agreement between the results of Hirschowitz *et al* and our own.

The inhibition of the response by atropine indicates that a cholinergic mechanism is critically involved. This was previously reported in the dog(1).

The response is mediated, at least in part, over the vagi because Hirschowitz *et al*(2) observed no response in vagotomized human subjects and Brodie(5) recently demonstrated that vagotomy abolished the response in esophageal-ligated and pylorus-ligated rats.

Summary. The gastric acid response to 2-D.G. (25-400 mg/kg) was studied in 7 chronic gastric fistula rats. The gastric response was "all or none" and the threshold dose was approximately 50 mg/kg. Atropine abolished the response. No dose of 2-D.G. produced a significantly larger acid response than insulin.

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Preparation of Inactivated St. Louis Encephalitis Virus Vaccine from Hamster Kidney Cell Culture.* (31456)

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The possibility of developing a vaccine against Japanese B encephalitis (JBE) and St. Louis encephalitis (SLE) was explored by Sabin *et al* in 1943(1). They developed mouse brain formalin inactivated vaccines which were capable of producing neutralizing antibodies in about 50% of the adult vaccinees. The SLE vaccine was never produced and used on a large scale for field testing as was the JBE vaccine. With tissue culture techniques available and the apparent recent success in developing an improved inactivated JBE vaccine in hamster kidney cell (HKC) monolayers(2), the possibility of developing a similar vaccine for SLE was explored.

Materials and methods. Virus strain used. (1) The large plaque variant(3) of P-15 strain, isolated from *Culex nigripalpus* mosquitoes of Pinellas County, Florida(4) was used. The first plaque picking (chick embryo tissue) was made from mouse passage 6, followed by 7 serial selections of large plaques. A pool was then prepared from this seed which was used for preparing the vaccine and for neutralization tests. (2) A 20% suspension of suckling mouse brain infected with mouse

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passage 3 of the P-15 virus was used for challenging the mice in potency tests.

Preparation of virus suspension. The virus was grown in primary HKC monolayers in the presence of maintenance medium containing 4% normal calf serum (NCS) in a method similar to that described for JBE virus(5). When cytopathic effect (CPE) started to appear, the medium was decanted, the cell sheets were washed 3 times with Hanks' balanced salt solution (BSS), then medium 199 containing 2% human albumin (H.Al.) was added, to be harvested about 16 hours later.

Formalin inactivation. The harvested virus-containing medium was centrifuged in the cold at 1000 $\times$ *g* for 30 minutes and the supernatant employed. Some lots were filtered through a Millipore disc of 0.22 μ pore size prior to addition of formalin. The pH of the virus suspension was measured, adjusted if necessary, formalin added by volume to a final concentration of 1:4000 at room temperature, and the contents of the vessel were then well mixed. Inactivation was carried out in a 37°C water bath with periodic removal of samples. These and a preinactivation sample were frozen and stored at —70°C until titrated. The inactivation reaction was drawn graphically.

Virus assay. Infectivity titrations were carried out by inoculating 10-fold dilutions of virus, each in 4 HKC tubes and endpoints were calculated by the Reed and Muench method(6). It had been shown previously for JBE virus that neutralization of residual formalin was not necessary(2). Hemagglutinin titers were determined by using the method of Clarke and Casals(7). Serial 2-fold dilutions were made in lucite plates and tested with equal amounts of 0.4% goose erythrocytes at the optimum pH (6.0 to 6.2) and at room temperature.

Potency tests. Immunogenic potency tests were carried out as with JBE vaccine(2) in a test designed by Sabin(1) as follows: (1) mouse potency test: serial 4-fold dilutions of vaccine (1/20 to 1/1280) were inoculated intraperitoneally (i.p.) in 1.0 ml amounts in groups of 12 mice in each of 2 doses, 3 days apart. One week from the first dose, the vaccinated mice and 24 controls of the same age were challenged each with 0.3 ml of a 10^{-1} dilution of suckling mouse brain virus. The challenge virus used had an i.c. LD_{50} titer of $10^{-8.5}$ or more in mice and killed at least 92% of the control mice. The mice were observed for 21 days and the minimal immunogenic dose (MID) of the vaccine required to protect 50% of the mice against the challenge inoculum was calculated by the Reed and Muench formula.

(2) Antigen extinction limit titer in guinea pigs. Serial 4-fold dilutions of vaccine (1/4 and 1/256) were each injected intramuscularly (i.m.) in three 1.0 ml doses, one week apart, into groups of 6 guinea pigs (450 g). Each guinea pig serum obtained 2 weeks after the last vaccine dose was mixed with an equal amount of virus containing about 100 $TCID_{50}$/0.1 ml, incubated at 37°C for 1 hour and each mixture inoculated in 0.2 ml amounts into 2 HKC tubes. Neutralization was considered to have taken place if one or both tubes did not show CPE. By applying the Reed and Muench formula, the extinction limit was calculated as the log of the reciprocal dilution of the vaccine producing antibodies in 50% of the guinea pigs, according to the method of Gard *et al*(8).

Experimental results. Virus yield in medium 199 containing 2% H.Al. Two separate experiments were carried out to explore the virus titers in medium 199 containing 2% H.Al. under different conditions. In the first experiment fluid harvests were obtained in media adjusted to pH 7.0 and 8.0 at the time of addition. In the second experiment, an additional variable, *viz.*, the "complete" harvest (homogenate of cells and fluid), as well as the fluid alone, both at pH 8.0 were tested.

The results of the 2 experiments (Table I) show that the pH 8.0 fluid harvest had a higher titer than that at pH 7.0 and that the complete harvest at pH 8.0 probably contained more virus than did the fluid alone. Both findings were similar to those for JBE virus(5).

Preparation and potency testing of 2 lots of formalin inactivated vaccine. The first lot of vaccine was prepared from a fluid harvest of virus in medium 199 containing 2% H.Al. at pH 7.0. The harvest was centrifuged, the pH of the supernatant adjusted to 7.1, formalin added to a final volume of 1:4000 and

TABLE I. Infectivity and Hemagglutinin Titers of St. Louis Encephalitis Virus Harvests in Medium 199 with 2% Human Albumin Under Different Conditions.

Exp No.	Type of harvest	Infectivity titer /.1 ml	HA titer /.4 ml
1	(1) Fluid harvest at pH 7.0	$10^{8.0}$*	128†
	(2) " " " pH 8.0	$10^{9.0}$	512
2	(1) Fluid harvest at pH 7.0	$10^{7.0}$	16
	(2) " " " pH 8.0	$10^{8.0}$	256
	(3) Complete harvest at pH 8.0	$10^{8.3}$	256

* Log_{10} of $TCID_{50}$ as calculated by Reed and Muench method.

† Reciprocal of highest dilution giving complete agglutination.

inactivation was carried out at 37°C for 71 hours. All samples before and during inactivation were titrated for infectivity only. The preinactivation titer was $10^{-7.3}$/0.1 ml and the inactivation course was biphasic with the inflection occurring early in the reaction (Fig. 1). The curve intercepted the abscissa at about 27 hours indicating that the total inactivation period was slightly more than 2½ times that needed for a "single" period. The mouse potency test gave a MID of 0.0079 ml and the challenge virus used had an i.c. LD_{50} of $10^{-8.9}$ in mice and killed 92% of the controls.

The second lot of vaccine was prepared from the homogenized "complete" virus harvest in medium 199 containing 2% H.Al. at pH 8.0. The centrifuged harvest was filtered and inactivation with formalin was carried out for 76 hours. Titration revealed that the prefiltration infectivity and HA titers were $10^{-9.0}$/0.1 ml and 1:256/0.4 ml, respectively. Filtration resulted in 0.3 log loss in infectivity without any change in HA titer. The inactivation curve was similar to that at pH 7.0, but the rate appeared to be faster at pH 8.0 (Fig. 1) and the "single" inactivation period was about 18 hours, indicating that the total inactivation period of the virus suspension was slightly more than 4 times that required for the curve to intercept the abscissa. The potency was tested in both mice and guinea pigs and results were as follows:

1. The MID was <0.0015 ml (only 3 of 12 mice inoculated with the 1/1280 vaccine dilution died, whereas all the others survived the challenge). The challenge virus had an LD_{50} of $10^{-8.7}$ i.c. in weanling mice and it killed 92% of the control unvaccinated mice.

2. The antigen extinction limit titer also could not be calculated from the customary dilutions used since all the guinea pigs given even the highest dilution of vaccine (1:256) showed antibodies in their sera to 600 $TCID_{50}$ of virus as titrated simultaneously in the test.

When 1.0 ml of this last lot of vaccine was tested in HKC for any live particles, results were negative in the primary culture and a blind subculture. Also, 2 litters of suckling mice inoculated i.c. survived the 21-day period of observation.

Discussion. When Sabin *et al*(1) studied the feasibility of developing an inactivated SLE vaccine from infected mouse brain, the potency of the best lots in mice ranged from a MID of 0.01 to 0.003 ml by using the Webster No. 3 strain of St. Louis virus. This strain was selected from 3 tested strains on the basis of its high titered yield in weanling mouse brain ($10^{8.2}$ to $10^{8.6}$ LD_{50}), and its apparent better antigenicity.

In the study reported here a plaque selected clone of the P-15 strain was the only one tested. It was available in low mouse passage and it grew in HKC to a reasonably high titer. Similarities of behavior during growth and inactivation, to the OCT-541 strain of JBE virus, were conspicuous. When virus was grown in HKC without serum by the same method used for the preparation of JBE vaccine (Darwish and Hammon 1966), a titer of $10^{-8.0}$/0.1 ml or more was obtained at pH 8.0 and the "complete" harvest at that pH seemed to have some advantage over the fluid alone. A direct relationship between HA and infectivity titers

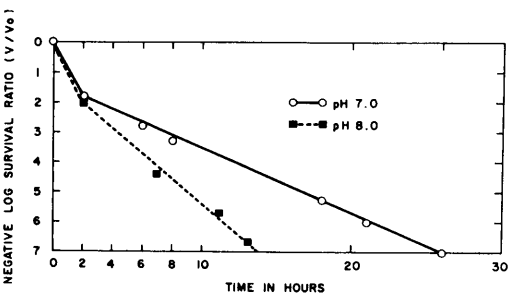

FIG. 1. Inactivation by 1:4000 formalin of St. Louis encephalitis virus in medium 199 containing 2% human albumin at pH 7.0 and pH 8.0, at 37°C.

as had been demonstrated previously with JBE virus(5) gave evidence of serving to estimate infectivity before proceeding to inactivation. The formalin (1:4000) inactivation curves showed that the virus was rapidly inactivated at 37°C. At pH 8.0 the reaction appeared to proceed at a faster rate than that at pH 7.0 as was the case with the OCT-541 wild strain of JBE virus and as had been reported with poliovirus(9). Loss in infectivity by filtration through a Millipore membrane also closely paralleled that of JBE virus, also the failure to detect any loss in HA titer(10).

For potency testing of the formalin inactivated tissue culture preparation the mouse test for MID was applied, as had been used for the JBE tissue culture vaccines and was also applied by Sabin *et al* to the early mouse brain St. Louis vaccine(1,2). A very low MID value (indication of high potency) was obtained. The first preparation, which had a preinactivation titer of $10^{-7.3}$/0.1 ml, gave a MID of 0.0079 ml which was within the range obtained by Sabin *et al* for the crude mouse brain vaccine(1). Preparation of a similar infective titer of JBE virus had given poorer MID titers of about 0.03 ml. This lot of SLE vaccine was inactivated for a total time slightly less than the "triple" period which was intended. However, in the second lot, prepared by improved methods, and with a preinactivation titer of $10^{-8.7}$/0.1 ml, the potency of the vaccine increased so much that the endpoints were missed using the standard range of dilution adopted for JBE vaccine. This high potency was obtained despite the fact that the lot was inactivated for more than 4 × that required for the inactivation curve to intercept the abscissa.

This vaccine has not been tested in man since it was made with a virulent virus and we consider that large amounts of final product should be employed in safety testing before each lot is tested in human volunteers. Our present facilities do not allow for such quantity.

If one can extrapolate the experience we gained with inactivated JBE vaccine prepared from a highly attenuated strain by the same method, and from Sabin's mouse brain vaccine tested in man, one should expect this St. Louis inactivated tissue culture vaccine to elicit a high level of neutralizing antibodies in man.

Before a vaccine such as this is given to man, in addition to the large amounts indicated above to be tested for freedom of detectable live St. Louis virus after and during inactivation and for adventitious agents before and after, it should be shown insofar as possible that the inactivation curve in its second segment continues as a straight line at least to some greater extent beyond the intercept than has been explored to date. This will require testing larger quantities or standard quantities of effectively concentrated materials. At present, it can only be assumed that this plotted rate of inactivation is constant after the intercept.

The question of the need of a St. Louis encephalitis virus vaccine, except for persons at greatly increased risk who work with it in laboratories, is open to question. A few endemic areas in the West where SLE virus was responsible for several cases and usually deaths as well, annually, such as the Yakima Valley in Washington(11) and the lower San Joaquin Valley(12) in California, have become relatively free of the disease due to intensive purposeful or accidental control measures affecting *Culex tarsalis* populations (13,14). Large urban outbreaks due to *Culex quinquefasciatus* transmission such as that of St. Louis and those of many smaller communities and more recently those of Houston, Texas and Camden, N. J., and to *Culex nigripalpus* in the Tampa Bay area of Florida are sporadic and as yet unpredictable. A constant, mass immunization program for these communities would appear to be unwarranted and after recognition of an outbreak a mass vaccination program might well be too late to prevent more than a few late cases. Despite the difficulties of prediction and of adequately rapid administration for epidemic use, a vaccine should be available for workers engaged in laboratories involved in handling group B arboviruses. Furthermore, one more vaccine for group B, added to yellow fever, dengue and JBE may afford considerable protection to other group B viruses beyond the homologous agent as has been demonstrated

repeatedly in this laboratory and in several others, insofar as laboratory animals are concerned. In such a case certain military, diplomatic, Peace Corps, industrial and other groups exposed in areas with endemic group B arboviruses might well utilize such a vaccine together with others in group B for the postulated breadth of at least partial protection against members of group B spread widely throughout the world. Furthermore, since JBE and SLE can apparently be prepared so easily by this means, a number of other group B viruses can probably be handled in the same way, especially those of the same subgroup, including those of Murray Valley encephalitis, West Nile, Ilheus, and bat salivary gland.

Summary. The large plaque variant of the P-15 strain of SLE virus which grows to a titer of 10^8 to 10^9 $TCID_{50}$/0.1 ml in HKC was inactivated by formalin and tested for antigenicity. The virus was harvested in medium 199 containing 2% H.Al., and was readily and apparently predictably inactivated by 1:4000 formalin at 37°C. Loss by filtration prior to activation was in the expected range of 0.3 log. The inactivated preparation when tested for the minimal immunogenic dose (MID) in mice gave a low value, indicative of excellent immunogenicity. The MID for a preparation with a preinactivation titer of $10^{-7.3}$/0.1 ml was 0.0079 ml, whereas that with a titer of $10^{-8.7}$/0.1 ml furnished an MID of <0.0015 ml. The latter vaccine elicited an unexpectedly good response in guinea pigs in an antigen extinction test. The protection the mice developed from such vaccine exceeded that of an earlier mouse brain St. Louis virus vaccine and that of a recently developed HKC JBE vaccine.

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Effect of L-Cystine and Sulfated Polysaccharides on Replication of Echovirus Type 32 in Monkey Kidney Cells.* (31457)

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In studies with recently isolated and characterized echovirus type 32 attempts to produce plaques by methods utilized were unsuccessful(1,2). Branche(1) noted that plaques could be produced when $NaHCO_3$ was incorporated into the overlay medium. This method was tested in our laboratory; plaques less

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