Grant and McLimans.[5] In both of these reports PABA was given only by mouth, in the acid form. In the experiments of Grant and McLimans the concentration of PABA in the diet was roughly one-tenth the amount which proved successful in our experiments. Indeed, it is surprising that any effect was noted with such a small amount of drug.

It is our opinion that a thorough clinical trial of PABA against tsutsugamushi disease in humans is justified by the definite effect which PABA exerts on experimental tsutsugamushi disease in gerbilles. This favorable effect has been fully verified by repeated trials, using strains of *R. orientalis* from widely separated regions (India, Ceylon, New Guinea). Furthermore, the clinical evidence from PABA therapy of epidemic louse-borne typhus[6] has shown that it is safe to give PABA to man in large amounts.

Summary. Additional evidence has been cited to indicate that the sodium salt of p-aminobenzoic acid definitely reduced the mortality of experimental tsutsugamushi disease in gerbilles. A description has been given of the routine of administration of Na PAB which was successful. Data from other, less successful, routines have been reported to emphasize the importance of the mode of therapy in the evaluation of PABA in experimental infection with *R. orientalis*. A clinical trial of PABA in human tsutsugamushi disease is strongly recommended.

[5] Grant, C. W., and McLimans, W. F., Report No. 2 (from the Naval Medical Research Institute, Bethesda, Maryland), 11 November, 1944.

[6] Yeomans, A., Snyder, J. C., Murray, E. S., Zarafonetis, C. J. D., and Ecke, R. S., *J. A. M. A.*, 1944, **126**, 349.

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Certain Bacteriostatic Agents Added to Sera Used in Diagnostic Tests for Neurotropic Virus Infections.*

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During the past five years a large variety of serological tests for virus infections, usually *in vivo*, but sometimes *in vitro*, have been made in this laboratory on several thousand serum specimens. Sera were generally tested for mouse protection against the viruses of both Western equine and St. Louis encephalitis and many were tested against the Lansing strain of poliomyelitis virus (adapted to the mouse by Armstrong[1]). Occasionally tests were made against the Eastern equine encephalomyelitis virus, the Japanese B encephalitis virus, a recently isolated strain of virus from California with neurotropic tendencies[2] and, more rarely, against still other neurotropic viruses. In addition to these tests, many of the specimens were subjected to experimental *in vitro* tests.

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[1] Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 1719.

[2] Hammon, W. McD., and Reeves, W. C., unpublished data.

TABLE I.

Experiment 1. The Effect of Zephiran 1:10,000, Merthiolate 1:10,000, and Sodium Sulfathiazole 1:500, as Serum Preservatives, on the Virus of St. Louis Encephalitis Under the Conditions of a Neutralization Test.

Bacteriostatic agent	Virus dilution				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Test of 1-11-44					
Zephiran	6/6*	5/6	4/6	0/6	0/6
Merthiolate	5/6	5/6	4/6	0/6	1/6
Sod. Sulfathiazole	6/6	6/6	4/6	1/6	0/6
Control	6/6	6/6	3/6	1/6	0/6
Test of 2-1-44					
Zephiran	6/6	6/6	6/6	4/6	0/6
Merthiolate	6/6	6/6	6/6	4/6	1/6
Sod. Sulfathiazole†	3/3	4/4	4/4	1/3	1/5
Control	6/6	6/6	5/6	3/6	0/4

* The numerator indicates the number of mice which died and the denominator the number inoculated in this and the following tables.

† Solution of Sod. Sulfathiazole had become toxic from holding for 20 days and several mice developed convulsions and some died soon after inoculation.

Sera for the most part, have been stored at about 5° C. As a result of repeated handling, and sometimes careless handling, contaminated sera frequently have been encountered. This has happened often enough to make it imperative to find some bacteriostatic agent to control the contamination without inactivating the viruses employed. Several agents were tested before one was finally rather arbitrarily selected for regular use.

Methods and Materials. After the desired quantity of the bacteriostatic agent had been added to normal rabbit serum, the serum was mixed and "incubated" with a virus suspension exactly as it would be in the course of a routine neutralization test. When the study was begun the technic of the neutralization test was that described by Hammon and Izumi.[3] Tubes of serum and virus were held at 5° C for five hours before inoculation into mice. Later, when it was noted in earlier literature,[4] and confirmed in our laboratory, that more effective neutralization occurred in the intracerebral test after incubating immune serum-virus mixtures for 2 hours at 37° C, the sera containing the bacteriostatic agent was incubated with the virus in this way before inoculation.

Five or ten fold serial dilutions of each virus were prepared and the range of dilutions so planned that all mice in the controls would die after inoculation with at least one or two of the lower dilutions, and few or none would die after inoculation with the highest dilution. Ampoules of frozen mouse-brain-virus suspension were used which had been previously titrated under similar circumstances. The tubes containing the serum-bacteriostatic agent-virus mixture to be inoculated, each contained 0.2 ml of serum including the bacteriostatic agent at the stated dilution, and 0.2 ml of a virus suspension. Thus, the bacteriostatic agent, in one half of its previous concentration in serum, was left in contact with the virus for either 5 hours at 5° C, or 2 hours at 37° C, prior to inoculation. As a control, serum to which saline had been added in the same amount as the bacteriostatic agent in the other tubes was similarly tested with the same serial dilutions of virus.

Zephiran, Merthiolate and Sodium Sulfathiazole. Experiment 1 (St. Louis virus).

Two tests were made with the St. Louis virus using Zephiran (Alkyl dimethyl benzyl ammonium chloride) in a 1:10,000 final dilution in the serum, Merthiolate, 1:10,000 and sodium sulfathiazole, 1:500 (0.2 per cent). These mixtures were held for 5 hours at 5° C. Results are shown in Table I. It

[3] Hammon, W. McD., and Izumi, E., *J. Immunol.*, 1942, **43**, 149.

[4] Cox, H. P., and Olitsky, P. K., *J. Exp. Med.*, 1936, **64**, 217.

TABLE II.

Experiment 6. The Effect of Phenyl Mercuric Borate, 1:50,000, as a Serum Preservative on the Virus of St. Louis Encephalitis Under Conditions of a Neutralization Test.

Bacteriostatic agent	Virus dilution			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Test of 5-23-44				
Phenyl Mercuric Borate	6/6	6/6	3/6	0/6
Controls	6/6	5/6	3/6	0/6

TABLE III.

Experiment 7. The Effect of Phenyl Mercuric Borate, 1:50,000, as a Serum Preservative on the Virus of Western Equine Encephalitis Under Conditions of a Neutralization Test.

Bacteriostatic agent	Virus dilution			
	10^{-7}	5×10^{-7}	25×10^{-7}	125×10^{-7}
Test of 5-23-44				
Phenyl Mercuric Borate	6/6	4/6	1/6	0/6
Controls	4/5	5/6	1/6	1/6

TABLE IV.

Experiment 8. The Effect of Phenyl Mercuric Borate, 1:50,000, as a Serum Preservative on the Virus of Poliomyelitis (Lansing Strain Mouse-adapted) Under Conditions of a Neutralization Test.

Bacteriostatic agent	Virus dilution		
	25×10^{-1}	25×10^{-2}	25×10^{-3}
Test of 10-17-44			
Phenyl Mercuric Borate	10/10	6/10	1/10
Controls	9/10	7/10	2/10

will be noted that none of the agents used had any apparent effect upon the virus. Sodium sulfathiazole, however, was unstable in solution and became toxic after standing for a few days, for a number of the inoculated mice developed convulsions, and some died. Following this experience and that of Experiment No. 2, performed on the same day, sodium sulfathiazole was not used again.

Experiment 2 (Western equine virus)

Two tests were made against the Western equine virus with Zephiran, and one with Merthiolate and sodium sulfathiazole. These agents were used in the same concentrations in serum as in the previous experiment; Zephiran 1:10,000, Merthiolate 1:10,000 and sodium sulfathiazole 1:500. In the first experiment 5 fold dilutions were employed and in the second 10 fold dilutions. Results were similar to those obtained with the St. Louis virus and it was concluded that there was no significant effect upon the virus by any of these agents.

Mercuric Cyanide. Experiments 3 and 4 (St. Louis and Western equine viruses)

At the suggestion of Dr. Troy C. Daniels of the College of Pharmacy of the University of California mercuric cyanide was tested. A 1 per cent solution in saline was added to the serum to make a final dilution of 1:10,000. Two tests were made with the St. Louis virus, and two with the Western equine strain. The St. Louis virus was used in 10 fold dilutions and the Western equine in 5 fold dilutions. No significant difference was noted between the preservative and control groups, with either virus.

Experiment 5 (Poliomyelitis virus)

The effect of mercuric cyanide was next tested upon the poliomyelitis virus (Lansing strain). Ten fold virus dilutions from 2.5×10^{-1} through 2.5×10^{-4} were employed, and mercuric cyanide was added to the serum in a final concentration of 1:10,000. The virus-serum mixtures were incubated for 2 hours at 37° C. The mercuric cyanide had

no significant effect on the strain of poliomyelitis virus.

Phenyl Mercuric Borate. Experiment 6 (St. Louis virus)

The next drug to be tested was phenyl mercuric borate. It was used in a final dilution of 1:50,000 in serum. It was first prepared in a dilution of 1:2,500 in saline for addition to the serum. All mixtures were incubated for 2 hours at 37° C. It will be seen in Table II that the drug had no effect upon the virus of St. Louis encephalitis.

Experiment 7 (Western equine virus)

When phenyl mercuric borate was tested with the Western equine encephalomyelitis virus, no virucidal effect could be detected. The results of the test are presented in Table III.

Experiment 8 (Poliomyelitis virus)

Poliomyelitis virus (Lansing strain) was not demonstrably affected by phenyl mercuric borate after incubation for 2 hours at 37° C. The results of the test are presented in Table IV.

Routine use of Preservative. For a short period of time following the tests with mercuric cyanide 1:10,000, this chemical was added to all sera as they were received. It appeared to be perfectly satisfactory. However, as soon as the tests were completed with phenyl mercuric borate, this latter agent was arbitrarily chosen to be used. A 1:2,500 solution in saline was prepared which has been kept in stock in the laboratory. In practice, to any tube of serum to be stored, 1 drop of this 1:2,500 solution is added per estimated milliliter of serum. The sera are stored at about 5° C.

The solution has been taken into the field and added to many sera obtained from wild mammals, domestic mammals, wild birds and domestic fowl. Many of these were undoubtedly contaminated with bacteria before the phenyl mercuric borate was added, for they were frequently collected under very unsatisfactory conditions in the field. A supply of a 1:2,500 dilution of phenyl mercuric borate was sent to the Kern County General Hospital, from which our laboratory receives by mail a large number of serum specimens. In all, a number slightly in excess of 1,000 serum specimens have had phenyl mercuric borate added to them within the past 12 months. Among all these sera only 8 have been found unsuitable for use from the standpoint of bacterial contamination, even though many have been used repeatedly for many tests. This is in great contrast to our former experience, particularly in regard to specimens of blood collected from animals shot or caught in the field.

Sodium sulfathiazole has not been used because solutions of it are not stable. Zephiran was not employed for it was felt that it might possibly interfere with certain *in vitro* tests because of its effect upon surface tension. Merthiolate or mercuric cyanide might prove satisfactory, for they are stable, sufficiently soluble and in a short trial of the latter no contraindication was found. The final selection of phenyl mercuric borate was more or less arbitrary, but we were probably influenced somewhat by the fact that it is being used to such a large extent in the preservation of plasma and other biological products, and used in a higher dilution than the other agents. Mercuric cyanide has not had—to our knowledge—such an extensive previous application.

Although we have not tested the effect of these agents on other viruses of the same group, through controlled serial dilutions, we believe it is safe to conclude that in all probability any of these agents may be safely used in neutralization tests with the other viruses. A number of our sera which contain phenyl mercuric borate have been used in neutralization tests against the Japanese B virus, against the new California virus and against the Eastern equine virus. None has been found to show any protection.

We have not performed any complement fixation tests with the above mentioned viruses but with mumps-virus-antigen, complement fixation tests[5] have been made in our laboratory on a number of the sera to which phenyl mercuric borate had been added.

[5] Enders, J. F., and Cohen, S., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 180.

There was no apparent interference. Further tests along this line are indicated.†

Summary and Conclusions. 1. Zephiran and merthiolate 1:10,000 and sodium sulfathiazole 1:500 in normal rabbit serum had no virucidal effect upon Western equine and St. Louis encephalitis viruses as determined through serial virus dilutions under the holding conditions and dilution of a standard neutralization test.

2. Mercuric cyanide 1:10,000 and phenyl mercuric borate 1:50,000 in serum under closely comparable conditions did not affect either of the above viruses or the Lansing mouse-adapted poliomyelitis virus.

3. Phenyl mercuric borate 1:50,000 has been used over a period of one year in over 1,000 serum specimens which have been subjected to diagnostic tests. There has been no apparent interference. Many of these sera have been used repeatedly in neutralization tests against several neurotropic viruses and in experimental *in vitro* tests including mumps complement fixation tests. This agent has greatly reduced difficulties previously encountered due to contamination of sera during collection and routine handling.

Phenyl mercuric borate is therefore recommended for routine use as a bacteriostatic agent for serum specimens to be used in *in vivo* diagnostic tests for the neurotropic viruses. Further experimental trial is required before it can be recommended for all types of *in vitro* tests.

† Since writing this approximately 300 sera containing phenyl mercuric borate have been tested for complement fixing antibodies to the Western equine or the Japanese B viruses. In these tests and several negative and positive controls with larger amounts of the preservative no interference was detected.

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Propagation of Theiler's GD-VII Mouse Virus in Tissue Culture.

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Some years ago, various attempts were made* to cultivate monkey poliomyelitis (MV) virus in fluid cultures of monkey olfactory bulbs and sympathetic ganglia. Although this work gave promise in so far as the survival of the tissue elements was concerned, the results were otherwise disappointing. It was decided, therefore, to undertake the cultivation of other neurotropic viruses, preferably ones that could be carried in mice, in order to gain experience that might be applicable, eventually, to the cultivation of human poliomyelitis. Jungeblut and Sanders[1] reported the propagation in mouse embryo brain cultures of the SK monkey poliomyelitis virus that had previously been transmitted to mice by intermediary passage through cotton rats. The culture medium used by them consisted essentially of ox serum ultrafiltrate. The present communication deals with the propagation of Theiler's GD-VII virus[2,†] of mouse encephalomyelitis under a wide variety of special conditions.

Materials and Methods. After the virus had been carried for 20 culture passages, during which time several different sets of con-

* Unpublished experiments carried out in association with Dr. Howard A. Howe and Dr. Geoffrey W. Rake.

1 Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

2 Theiler, M., *J. Exp. Med.*, 1937, **65**, 705; Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49, 79.

† The virus was supplied through the courtesy of Dr. Max Theiler of the Laboratories of the International Health Division, The Rockefeller Foundation, New York.