11. ———, XXII Int. Cong. Physiol. Sciences, Leiden, Holland., 1962, v22, 79.
12. Russek, M., Fernandez, A. F., Vega, C., Am. J. Physiol., 1962, v204, 309.

Received March 15, 1967. P.S.E.B.M., 1967, v125.

A Plaque Neutralization Method for Arboviruses. (32194)

E. EARLEY,* P. H. PERALTA, AND K. M. JOHNSON
Middle America Research Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Balboa Heights, Canal Zone

The plaque technique developed by Dulbecco(1) provides a method for relatively precise infectivity assay of many animal viruses. Although several types of cell cultures have been tried in the past decade (2,3), successful application of this method to the study of arboviruses has been limited to certain members of groups A(4), B(5), and C(6).

Ideal objectives in the case of arboviruses include: discovery of a single cell culture system in which discernible plaques are induced by a wide variety of viruses and the demonstration that cell monolayers of very small size will provide sufficient quantitative accuracy to permit use of the plaque method for routine procedures which require many individual cultures. In the latter regard, encouraging results have been reported by Miura and Scherer(7) and by Rubio-Brito (8), in which individual arboviruses were successfully assayed and specifically neutralized in cell monolayers grown in the wells of leucite trays originally developed for studies on viral hemagglutination.

This communication describes successful production of plaques by many arboviruses from the western hemisphere in a "microsystem" utilizing disposable plastic trays initially used for performing metabolic inhibition tests with polioviruses(9).

Results obtained using this system for measurement of neutralizing antibodies to Venezuelan equine encephalomyelitis (VEE) virus are compared with those obtained in a mouse neutralization test and by hemagglutination inhibition.

Materials and methods. Tissue cultures. Several types of cell cultures were tested in preliminary trials but the MA-111 line of continuous newborn rabbit kidney† was selected because all of the 19 viruses screened produced plaques under agar. Except as specified, all experiments reported were carried out in this cell line. Recent experience suggests that a line of African green monkey (*Cercopithecus aethiops*) kidney cells (VERO) (10) may be superior to the MA-111 cell line. Virus titers were higher in certain instances and plaque resolution was often better. Methods used for preparation of both cell lines were the same.

Monolayer cell cultures grown in 32 oz ungraduated prescription bottles‡ were subcultured twice weekly. The growth medium (GM) consisted of medium 199§ containing 1.25 g of $NaHCO_3$ per liter, supplemented with 5% fetal calf serum, 100 units of penicillin and 100 μg of streptomycin per ml.

Preparation of cell cultures in trays. Cells were grown in two types of plastic trays manufactured by Linbro Chemical Co., New Haven, Conn. Wells in the model 96CV have a slightly concave bottom, provide a cell surface area of about 2.0 cm^2 and have a fluid capacity of 2.2 ml. Those of model 54FB are flat bottomed, with a surface area of 2.2 cm^2 and a capacity of 3.0 ml. Trays were cleaned and sterilized by 5 minutes' immersion in 95% ethanol, followed by exposure to ultraviolet light (GE 30 watt,

* Present address: Dept. of Pathology, School of Med., Tulane Univ., New Orleans, La.

† Microbiological Associates, Inc., Bethesda, Md.
‡ Brockway Glass Co., Brockway Pa.
§ 10 × Concentrate - Microbiological Associates, Inc., Bethesda, Md. Dry concentrate - General Biochemicals Co., Chagrin Falls, Ohio.

germicidal) for 16-18 hours at 17 inches distance. Clear styrene covers were similarly irradiated for 1 hour, then placed on the trays. Monolayers previously washed with Dulbecco's phosphate buffered saline(4) without Ca^{++} or Mg^{++} ions (DS), were harvested by placing 10 ml 0.25% trypsin in DS (pH 7.4) on the cell sheet for 1 minute, then decanting this solution. After 20 to 25 minutes at room temperature the detached cells were resuspended in GM. Cells were then enumerated and the suspension adjusted to contain 240,000 cells/ml. Wells of 96CV and 54FB trays were inoculated with 0.5 ml and 0.6 ml of this suspension, respectively. After 48 hours' incubation at 36°C in a humidified atmosphere containing 4% CO_2, uniform monolayers were ready for use.

Viruses. The arboviruses used for these tests were either prototype strains or isolates recovered at the Middle America Research Unit, Ancon, Canal Zone(11). Stock virus pools were prepared as 10% suspensions of infected mouse brains in 0.05 M borate-0.12 M NaCl, pH 8, containing 10 to 25% normal rabbit serum (heated 56°C-30′) and stored at —60°C.

Virus inoculum and overlay of cultures. The fluid medium was removed by inverting the trays and wiping off the outer surface with layers of sterile gauze. For virus titrations, serial 10-fold dilutions in tubes were made in phosphate buffered saline containing 0.5% gelatin, pH 7.1, (PBS-G). Approximately 0.05 ml (2 drops from Pasteur pipette) of each virus dilution was dispensed into each of 4 wells. Virus was allowed to adsorb for 1 hour at 33°C. Then 0.5 ml of overlay medium was added to each well using a Cornwall syringe. After the agar had hardened, cultures were incubated at 36°C in 4% CO_2 until appropriate time for the second, staining overlay medium. Depending upon the virus, this interval varied from 1 to 5 days. Each well received 0.5 ml of the same overlay containing 1:25,000 concentration of neutral red. Trays were reincubated and read for plaques 1-3 days later.

The overlay medium consisted of equal volumes of 2% agar (Difco-Bacto or Difco-Noble) autoclaved (15 lb, 15′) and held at 45°C, and of a solution containing the following pre-sterilized components: 56.7% distilled water; 20.0% 10×-concentrated, modified Earle's salt solution (NaCl 68.0, KCl 4.0, $MgCl_2 \cdot 6H_2 \cdot O$ 2.0, NaH_2, $PO_4 \cdot H_2O$ 1.32, glucose 10.0 $CaCl_2$ 2.0, phenol red 0.1 g/l, sterilized by means of Millipore GS filter); 13.3% yeast extract-lactalbumin hydrolysate;‖ 4.0% heated (56°C-30′) fetal calf serum; and 6.0% $NaHCO_3$ solution (7.5%); to which were added 2.0 ml stock penicillin-streptomycin solution¶ per 100 ml.

Neutralization tests. Serial 2-fold dilutions of sera or mouse ascitic fluids (heated 56°-30′) were made in PBS-G by the loop technique, in round bottom microtrays designed for complement-fixation tests(12). A virus suspension containing an estimated 80 to 100 plaque forming units (PFU) per 0.05 ml (based on previous titrations) was prepared in PBS-G, and an equal volume of this dilution was added to the volume of each serum dilution. Following incubation at room temperature for one hour, 0.05 ml of this mixture was inoculated into each well, using 2 wells per dilution. At this time a titration of the virus suspension (adjusted to compensate for absence of serum) was made, employing 3 four-fold dilutions and 4-6 wells per dilution. Eighty per cent reduction of plaques was taken as evidence of neutralization.

Results. Production of virus plaques. Each of 19 available arboviruses tested was found to produce plaques in MA-111 cells (Table I). Furthermore, virus titers as determined by plaque count closely approximated those observed when the same pools were assayed by intracerebral inoculation of infant mice. As indicated in Table I, there was considerable variation in time of appearance and size of plaques among the different viruses. The effects of DEAE-dextran (DEAE) or sodium dextran (NaDS) were noteworthy. Although Group A and

‖ 1% yeast extract and 5% lactalbumin hydrolysate previously autoclaved separately and combined in equal volume.

¶ 10,000 units penicillin G and 10,000 μg dihydrostreptomycin per ml.

TABLE I. Comparative Infectivity Assays of Various Arboviruses in Infant Mice and MA-111 Cell Cultures.

Group	Virus	Reciprocal $\log_{10}$ titer/ml in: Infant mouse	Cell culture	Temporal development, size, dextran effect		
A	EEE	9.3	8.3	1*	L†	—‡
	VEE	9.0	9.3	1	L	—
	Una	7.1	6.8	1	M	—
	Pixuna	8.5	8.5	2	M	—
	Mucambo	8.5	8.6	2	L	—
B	Ilheus	8.3	8.2	4	S	D
	Yellow fever	9.1	8.8	5	M	D
	SLE	10.2	9.2	4	S	D
	Bussuquara (GA7)	7.5	6.3	4	M	D
Bunyamwera	Guaroa (BT-1122)	6.1	6.6	4	S	D
	Cache Valley (BT-2368)	7.3	7.4	2	M	D
	Wyeomyia (BT-219)	5.6	5.3	7	S	D
California	B-878	7.1	6.6	6	S	D
	B-1113-14	7.3	6.3	5	S	D
Changuinola	BT-104	6.2	5.5	4	S	D
Sandfly fever	Chagres	6.6	6.2	5	S	NaDS
Vesicular stomatitis	New Jersey	7.0	6.8	1	VL	—
	Indiana	7.2	8.0	1	VL	—
	Cocal	8.7	8.6	1	VL	—

* No. days required for optimum plaque count.
† Plaque size: VL, >3 mm; L, 2-3 mm; M, 1-2 mm; S, <1 mm.
‡ D, plaque size and/or number enhanced by 100 μg/ml DEAE-dextran; NaDS, enhanced by 50 μg/ml sodium dextran sulfate; —, no effect of additives.

vesicular stomatitis virus (VSV) patterns were not significantly altered, the majority of other viruses produced more or larger plaques in the presence of 100 μg/ml of DEAE incorporated into the initial overlay. In at least one instance, strain B-878 belonging to the California encephalitis complex, DEAE was essential for plaque demonstration. In contrast, the recently recognized Chagres virus produced more distinct plaques in the presence of NaDS.

Some qualitative and quantitative parameters of plaque formation. Experiments designed to measure certain details of plaque formation were carried out using VEE and St. Louis encephalitis (SLE) viruses. Comparative assays in well and 60 mm plastic petri dishes revealed (Table II) that the former were 2-6 times less sensitive than dishes for measuring virus, a not unexpected finding in view of the relatively reduced area-to-circumference ratio of the wells. Depending on the virus in question the maximum number of plaques that could be counted accurately in wells ranged from about 15 to 30.

TABLE II. Comparative Infectivity Assays in Petri Dishes and Multiple-Welled Trays.

Dilution	No. of plaque forming units/0.05 ml*: VEE virus Dish†	VEE virus Well	SLE virus Dish	SLE virus Well
Initial†	373	c	167	c
1:4	201	c	88	60
1:16	94.3	29	57	28
1:64	35.5	6	12	6

* Average of 3 dishes of 21.2 cm^2 area and 12 wells of 2.0 cm^2 area.
† VEE virus $10^{-5.5}$, SLE virus, $10^{-5.0}$.
c = Plaques confluent.

Although adsorption experiments at 33°C and 36°C with VEE and SLE viruses indicated no significant difference in either virus titer or plaque size, the former was chosen in order to minimize evaporation from the cell sheet during this time. However, the most critical factor in preventing the cell sheet from sloughing from the plastic after the growth medium had been drained from the tray was the rapid inoculation of virus-serum mixture into the wells.

TABLE III. Effect of pH on Virus Adsorption.

pH*	No. of PFU† VEE	BT-104	pH*	No. of PFU† VEE	BT-104
6.6	18.0	47.0	7.4	29.3	80.0
6.8	22.3	79.6	7.6	16.0	68.3
7.0	21.3	117.5	7.8	5.3	42.6
7.2	34.8	69.6	8.0	1.0	48.0

* Dulbecco's buffered saline adjusted by reciprocal variation of Na_2HPO_4 and KH_2PO_4.
† Average of 3 60 mm petri dishes.

Experiments with these two agents as well as Chagres and Changuinola viruses showed that at least 85% of ultimate plaque number was achieved after 1 hour of incubation. Further work was therefore done employing 33°60′ for virus adsorption and every effort was made to inoculate an entire panel in not more than 10 minutes after GM had been discarded.

The effect of pH on virus adsorption is shown in Table III. Values between the limits of about pH 7.0 to 7.4 gave maximum plaque counts. Since it was not feasible to wash the trays prior to virus inoculation, PBS-G at pH 7.1 was used as diluent so that the small residue of GM plus the diluent would not exceed pH 7.4 during the period of virus adsorption. Using this technique replicate assays of VEE and SLE viruses were carried out in wells and, as shown in Table IV, were found to conform to a nearly regular Poisson distribution at several different dilutions.

Serial passage of cells did not appear to alter virus plaque patterns within the limits studied. It was regularly noted, however, that the metabolic rate of VERO cells, as measured by the rate of pH drop of GM, increased significantly with passage. This observation was clearly associated with increasingly poor definition of plaques in the case of some of the more fastidious viruses for which DEAE was originally found beneficial. For this reason, as well as that of progressive likelihood of contamination with mycoplasma, it was decided to limit the use of a given sub-line of VERO cells to 30 passages after initiation from a pool of frozen cells.

TABLE IV. Variation in Number of Plaque Forming Units Produced by SLE and VEE Viruses in Multiple Welled Trays.

Virus	Exp	No. of plaque forming units Mean*	Range	50% of wells
SLE	1	39.0	21-64	32-46
	2	37.8	15-64	30-44
	3	11.3	2-17	9-14
	4	16.5	9-30	13-17
	5	15.4	4-23	12-18
	6	5.2	0-12	3- 6
	7	5.8	1-11	4- 7
VEE	1	23.9	16-29	22-26
	2	18.1	12-25	18-22
	3	8.9	4-13	7-11
	4	8.7	5-14	7-10

* 40 wells each experiment.

The effect of neutral red on plaque formation was also investigated. It was found that cultures of both MA-111 and VERO cells could survive for several days in presence of as much as 1:40,000 final concentration of this dye. Incorporation of neutral red into the initial overlay medium, however, delayed plaque development and, for most viruses, also reduced the count and sharpness of definition of plaques. Detailed study of the phenomenon with EEE virus showed that it was not dependent on photoinactivation of virus by the dye. Whether the overlay procedure was carried out with the usual laboratory lighting or with infrared illumination only, addition of neutral red with the initial overlay led to smaller (less than 1 mm compared to 2 mm), less well-defined plaques and a plaque count 0.3 log lower, that reached its maximum later, than when neutral red was added in the second overlay at 24 hours.

In view of these findings, it was decided as a standard procedure, to use 2 successive agar overlays with the dye in the second. An exception to this practice, however, was subsequently made for VSV. Each of the 3 types produced such large and rapidly confluent plaques that counting was extremely difficult. Incorporation of neutral red reduced plaque size to such an extent that the number of plaques per well that could be accurately counted reached the 15-30 range of the other viruses.

Virus neutralization. Neutralization tests with hyperimmune mouse sera or ascitic fluids were done with 13 of the viruses. All

homologous titers were at least 1:64, and most between 1:512 and 1:4096. A hyperimmune VSV-NJ mouse ascitic fluid was found to have a titer of 1:80,000. Neutralization of plaques was complete with low serum dilutions and there was usually an "end-point" dilution containing a reduced, countable number of plaques.

The effect of unheated guinea pig serum on neutralization of VEE virus was determined. In preliminary experiments, 10% fresh serum incorporated into the diluent used for making ascitic fluid dilutions appeared to enhance the specific neutralization titer between 4-16 fold. Closer study, however, revealed that this apparent enhancement was due to non-specific plaque inhibition by the guinea pig serum. In 3 experiments, 10% guinea pig serum was found to reduce VEE virus titer 1.0, 1.2 and 1.3 logs, respectively. In a neutralization test in which the virus dose was adjusted to compensate for the non-specific reduction by guinea pig serum, the endpoint of specific neutralization by each of 3 human immune sera (1:64, 1:128, 1.256, respectively), was the same as when guinea pig serum was not included in the test. Furthermore, the non-specific activity of the serum was not abolished by heating at 56°C for 30′, and it was found that heat-stable plaque-reducing activity was also exhibited by a variety of animal sera (horse, calf, chicken). Subsequent neutralization tests were therefore done without supplementary serum.

A comparison of plaque neutralization, mouse neutralization index, and hemagglutination-inhibition (HI) methods for detection of VEE virus antibodies was made using 46 human sera obtained during a study of a population from which the virus had been recovered. Mouse and plaque neutralization results were qualitatively identical. Fig. 1 shows the complete qualitative and significant quantitative correlation obtained between plaque neutralization and HI tests.

Preliminary studies suggest that this system can be used also for the identification of viral isolates. For this purpose it was possible to titrate either the unknown virus or known serum in the presence of a fixed dilution of

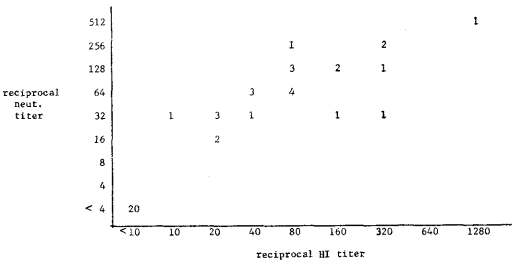

FIG. 1. Comparison of VEE virus antibody titers in 46 human sera by plaque neutralization and hemagglutination-inhibition techniques.

the reciprocal reagent. Results of an identification test with three closely related Group A arboviruses, including three strains of VEE virus, are shown in Table V. Viruses were titrated with and without prior incubation in a 1:10 dilution of hyperimmune VEE virus ascitic fluid.

Discussion. These results suggest that it may be possible to perform sensitive neutralization tests for a large number of different arboviruses in a single line of cultured cells. Although many experiments are needed before the method can be standardized for specific agents, the data obtained for VEE virus and the relative ease with which plaques were produced in quantitative correlation with mouse titrations for 19 viruses are highly encouraging. Should further work justify this optimism, the significant advantages of the technique over the "standard" mouse test include: a) a saving of at least 10-fold in volume of serum needed to obtain a quantitative result; b) avoidance of uncontrollable, usually ill-definable, variation in results obtained in animal tests occasioned by presence

TABLE V. Identification of Group A Arbovirus Strains by Plaque Neutralization Test.

Virus	Plaque forming units in presence of: Normal MAF*	VEE immune MAF (TC80)	Index PFU neutralized
VEE (TC80)	2.8×10^8	4.4×10^5	640
VEE (10858-H)	1.6×10^7	1.2×10^5	130
VEE (BT 2607-M)	2.4×10^8	2.4×10^6	100
Pixuna	8.8×10^6	3.6×10^6	2.4
Mucambo	6.0×10^7	2.8×10^7	2.2

* MAF = Mouse ascitic fluid.

in the animals of extraneous micro-organisms, whether pathogenic or clinically inapparent; c) sizable reduction in size of breeding colonies of animals necessary to support laboratories engaged in arbovirus research.

The ability of the present method to provide quantitative estimation of antibody by dilution of serum rather than virus confirms the initial observations of Muira and Scherer(7). The fact that addition of fresh serum containing "accessory factor", previously found to be important in virus-antibody reactions with certain other agents, did not enhance this reaction in the case of VEE virus was surprising. However, the work of Whitman(13) was done employing mice and that of Webb (14), utilizing cell cultures incubated in fluid medium. In contrast to the plaque method, both of these systems tend to produce a disproportionate reduction in detectable antibody titers when individual infectious virus particles become dissociated from antibody molecules. Although it has not been conclusively shown to our knowledge, we believe it likely that the potentiating effect of "accessory factor" is to exert an alteration of the kinetics of virus-antibody reaction in the direction of formation of the aggregated molecule. If this assumption is correct, "accessory-factor" would be of value in plaque neutralization tests only in those cases where the tendency for dissociation of the virus-antibody complex is extremely high.

The major unresolved problem appears to be that of potential "non-specific" virus inhibitors in individual sera of different animal species, such as were encountered in the experiments with VEE virus. Where dilution of serum, as practiced by Muira and Scherer in the case of Japanese B encephalitis virus(7) proves sufficient to eliminate false positive neutralization without significant reduction in test sensitivity, this appears to be the method of choice. In other instances, high incidence of significant non-specific activity in sera from various animal species may dictate the need to incorporate such serum into diluents and to readjust virus dose in the presence of this material so that only specific antibodies will be detected.

The satisfactory pattern of plaque production by a variety of viruses in either MA-111 or VERO cells also recommends renewed attempts to compare the sensitivity of these cell cultures with infant mice for primary isolation of arboviruses. Such efforts, however, would require use of dishes rather than the present wells in order to achieve maximum sensitivity.

Summary. A method for production of plaques by arboviruses in small wells of disposable plastic trays is described. Each of 19 viruses from Central or South America produced plaques in MA-111 and VERO cell lines. Optimum conditions for various agents were determined. The method was shown to give reliable measurement of VEE virus antibodies in sera from humans with naturally acquired infections.

Addendum. Since this manuscript was submitted, continuing experience has resulted in several modifications in technique which have been adopted as "standard" in this laboratory. These include: (1) Exclusive use of VERO cells for all viruses. These cells have been found to yield more cells per bottle ($20\text{-}40 \times 10^6$ *vs* $10\text{-}15 \times 10^6$), are easier to remove from glass surfaces, and in certain instances produce better plaque patterns than MA-111 cells. (2) Substitution of plastic disposable pipette tips (#05-688) for Pasteur pipettes to inoculate virus-containing suspensions. These short, uniform pipettes are manipulated with a non-electric automatic pipette (#05-719) using an adapter to deliver exactly 0.05 ml of fluid. This equipment was supplied by Becton-Dickinson Co., Rutherford, N.J. (3) Incubation of agar-overlayed panels in an ordinary incubator rather than in an atmosphere containing CO_2. This economy was made possible by altering the buffer system to contain 1.0 ml of 2 M Tris (Sigma 7-9)**, 0.5 ml of 400 mM stock of oxalacetic acid†† (stored at −60°C) and 1.1 ml 7.5% $NaHCO_3$/100 ml of agar-free medium.

The list of arboviruses successfully assayed in this system has steadily increased. Recent additions include: Western encephalomyelitis, Mayaro and Una of group A, dengue type 2 of group B, and Oriboca, Caraparu, Marituba

** Sigma Chemical Co., St. Louis, Missouri.

†† Nutritional Biochemical Co., Cleveland, Ohio.

and Nepuyo of group C.

1. Dulbecco, R., Proc. Nat. Acad. Sci., 1952, v38, 747.
2. Kissling, R. E., Proc. Soc. Exp. Biol. & Med., 1957, v96, 290.
3. Henderson, J. R., Taylor, R. M., J. Immunol., 1960, v84, 590.
4. Dulbecco, R., Vogt, M., J. Exp. Med., 1954, v99, 183.
5. Porterfield, J. S., Nature, 1959, v183, 1069.
6. Henderson, J. R., Taylor, R. M., Proc. Soc. Exp. Biol. & Med., 1959, v101, 257.
7. Miura, T., Scherer, W. F., Am. J. Hyg., 1962, v76, 197.
8. Rubio-Brito, R., Rev. Latinoamer. Microbiol., 1962, v5, 81.
9. Rightsel, W. A., Schultz, P., Muething, D., McLean, I. W., Jr., J. Immunol., 1956, v76, 464.
10. Simizu, B., Rhim, J. S., Wiebenga, N. H., Bact. Proc., 1965, p112.
11. Peralta, P. H., Shelokov, A., Am. J. Trop. Med. & Hyg., 1966, v15, 369.
12. Sever, J. L., J. Immunol., 1962, v88, 320.
13. Whitman, L., ibid., 1947, v56, 97.
14. Webb, P. A., Am. J. Trop. Med. & Hyg., 1965, v14, 790.

Received March 16, 1967. P.S.E.B.M., 1967, v125.

Analgetic Activity of a Quinazoline. (32195)

H. I. CHERNOV, H. M. BLATTER, P. S. BERNARD, D. E. WILSON AND A. J. PLUMMER (Introduced by F. F. Yonkman)
Research Department, CIBA Pharmaceutical Co., Summit, N. J.

Recently, a series of quinazoline compounds was found to have analgetic activity that may be characterized as non-narcotic and unique. One of these, 4-(2-dimethylamino-ethylamino)-quinazoline (Fig. 1), designated Su-13026, is the most interesting and its pharmacology will be described here.

Materials and methods. Drugs were prepared as aqueous solutions just prior to testing. Doses given were based on the salts. The following compounds were used: morphine sulfate, aminopyrine, d-amphetamine sulfate (Dexedrine),* d-propoxyphene hydrochloride (Darvon)† and nalorphine hydrochloride (Nalline).‡

Male albino mice (CF-1) weighing 18 to 22 g were used. The ED_{50} values were based on at least 3 logarithmically spaced (0.3 interval) doses per drug. The doses utilized were those for which the range of responses in preliminary tests fell between 16 and 84% (probits 4.0 and 6.0). The best-fitting straight line was determined on logarithmic-probability paper by the method of Litchfield and Wilcoxon(1). All drugs were given orally or subcutaneously at a concentration adjusted for an injection of 0.20 ml per animal.

A constant intensity heat stimulus was used to induce a tail-flick response(2). The apparatus previously described(3) was modified in that a light source with a built-in parabolic reflector (Sylvania, T-12, 150 watts) was focused from a point 5 cm below the tail of the mouse.§ The control reaction time was measured twice in each of 10 animals. To standardize the procedure, the intensity of the stimulus was adjusted so that the control values were between 3.5 and 4.5 seconds. Thus, in a series of 16 experiments the mean ($\pm$ S. D.) control value was 3.95 $\pm$ 0.33 seconds. At each post-drug interval those animals that had a reaction time greater than 1.32 plus the average control value for the group were considered to be "reactors" ($P = <0.001$). The number of reactors at the time of peak drug effect was converted to a per cent value and treated as described above.

For the interaction studies, paired experiments were done using the tail-flick procedure. The test compound and nalorphine 5 mg/kg or amphetamine 5 mg/kg were given to the first group of 10 mice. The second group re-

* Smith, Kline and French Laboratories, Philadelphia, Pa.

† Lilly Laboratories, Indianapolis, Ind.

‡ Merck, Sharp and Dohme, West Point, Pa.

§ This unit is commercially available from W. A. Schaerr, Brooklyn, N. Y.