AN EFFECTIVE ANTIVIRAL SYNTHETIC*

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Specific therapeutic agents against true animal or human viruses have not heretofore been available. Although recent reports indicate that therapy against the members of the lymphogranuloma-psittacosis group is a likelihood in the near future, it is generally agreed that such viruses as trachoma, inclusion blennorrhea, psittacosis, lymphogranuloma venereum and meningopneumonitis are more closely related to the rickettsiae than to such agents as herpes simplex, equine encephalomyelitis, poliomyelitis, etc.

The present communication is a general statement of the successful use of a chemotherapeutic agent against a mouse virus. Detailed data on numerous experiments involving thousands of mice and more than a hundred monkeys will be published at a later date.

In 1947 a large number of compounds were tested at the Lederle Laboratories for possible activity against an experimentally induced infection with a strain of western equine encephalomyelitis virus. The activity of these compounds was determined by treating mice infected at least 48 hours prior to receiving drug treatment. Among these compounds a new sulfonamide synthesized at the Calco Chemical Division of the American Cyanamid Company showed promise. The compound, N-(2-thiazolyl)-phenol sulfonamide, was named pheno-

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sulfazole.[1] In September, 1947, permission was received to test this drug against poliomyelitis.[2]

The Test Virus

The strain of virus selected as the infection to be treated was a mouse virus originally isolated in 1940. It was used throughout the present study for the following reasons:

1. It is infectious for mice in high dilutions by all routes of inoculation.
2. It is a stable and predictable virus, producing encephalitis and paralysis in mice.
3. It can be grown in tissue culture with ease and in this milieu produces an infection in mice predominantly paralytic in character.

In order to obtain a fairly standardized seed virus, the supernate of a fluid tissue culture was injected intracerebrally into 200 Swiss mice weighing approximately 10 to 12 grams. As symptoms of encephalitis and paralysis appeared, the brains of 72 moribund animals were pooled. After emulsification in a Waring blender the mouse brain pool, diluted by weight to 20% with a mixture of 10% horse serum in Simms salt solution, was distributed in vials and stored in CO_2 ice. During the past 9 months, this pool has consistently yielded titers of approximately 10^{-7} in mice infected via the intraperitoneal route.

The Drug

Phenosulfazole, N-(2-thiazolyl)-phenol sulfonamide, is a white powder that goes into solution with difficulty. Since it became apparent in the early experiments that the powder was not efficiently absorbed, a sodium salt of the drug was made. This salt has proved to be soluble, non-toxic and generally satisfactory as an injectable material. The results in mice

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[1]Trade name Darvisul.

[2]We wish to express our deepest appreciation to Mr. Fritz Popken and his associates for placing their data at our disposal prior to publication and to Dr. M. E. Hultquist and his associates for making available a supply of the drug.

summarized in Table I have been obtained with the use of the sodium salt.

Schedule and Course of Treatment in Mice

Various schedules of treatment were investigated in the early experiments. For present purposes, it is sufficient to say that the schedule now used in this laboratory is as follows:

Swiss mice weighing 16 to 20 grams are injected intraperitoneally with 0.06 ml. serial dilutions of the mouse brain pool. The groups of mice receiving the range of dilutions of virus within which it is desired to attempt treatment (usually $2x10^{-4}$, $2x10^{-5}$, $2x10^{-6}$, $2x10^{-7}$) receive the first injection of drug 24 hours after the intraperitoneal injection has been given. Once treatment has been initiated, each mouse receives 4 daily injections, at 8 A.M., 12 NOON, 3 P.M., and 6 P.M.

At 8 A.M. 0.5 ml. of drug is given and at 12 NOON and 3 P.M. the dose is 0.25 ml. At 6 P.M. 1.0 ml. of drug is injected. Thus each animal receives a total daily fluid injection of 2.0 ml. Since the concentration of drug has been either 4 or 8 mgms. per ml., the daily intake of drug per animal is either 8 or 16 mgms. This schedule is maintained for five days.

In a recent experiment involving 300 mice the treatment schedule was altered so that a daily total of 16 mgms. of drug was given in two injections, one ml. containing 8 mgms. at 8 A.M. and another at 6 P.M. The results suggest that this schedule is at least as effective as a course of treatment given four times daily (Exp. F, Table I).

It is to be emphasized that large mice (at least 16 to 20 grams) have been found to be better experimental subjects. Indeed the slight irregularities noted in Experiments A, B and C (Table I) were found to be due to smaller mice. When animals weighing less than 15 grams were eliminated from the experiment, the results were more consistent (Exp. D, Table I).

It should also be noted that treatment was initiated 24 hours after infection for the following reason: In the original studies with this agent it was found that the virus was unusually invasive once it was placed in the peritoneal cavity. Indeed, within 2 hours after intraperitoneal injection, the virus could

TABLE I

ACTIVITY OF SODIUM PHENOSULFAZOLE

A summary of experiments in which treatment was started 24 hours after a pool of mouse poliomyelitis virus was inoculated intraperitoneally into the mice. Results on Fifth and Fourteenth Days after Infection

Experiment	Results on	Total Dose of Drug in Milligrams	SURVIVORS/TOTAL in Dilutions of Virus Tested				% Survivors (14 Days)	Final Titer (14 Days)
			10^{-4}	10^{-5}	10^{-6}	10^{-7}		
A	5th Day	35	15/20	19/20	14/20	18/20		
	14th		6/20	17/20	11/20	17/20	63	$10^{-4.8}$
	5th Day	0 (controls)*	0/20	2/20	0/20	14/20		
	14th		0/20	0/20	0/20	12/20	15	$10^{-6.8}$
B	5th Day	80	16/18	8/18	10/18	18/18		
	14th		9/18	7/18	8/18	14/18	52	$10^{-5.5}$
	5th Day	40	14/18	12/18	14/18	12/18		
	14th		8/18	7/18	10/18	11/18	50	$10^{-5.7}$
	5th Day	0 (controls)	0/18	0/18	3/18	10/18		
	14th		0/18	0/18	0/18	8/18	11	$10^{-7.0}$
C	5th Day	40	6/10	7/10	6/10	8/10		
	14th		4/10	6/10	5/10	8/10	57	$10^{-5.1}$
	5th Day	0 (controls)	0/10	0/10	3/10	4/10		
	14th		0/10	0/10	0/10	4/10	10	$10^{-7.0}$

*Control animals received intraperitoneal injections of 0.85% saline comparable in regard to volume of fluid and schedule with mice under treatment. Additional controls consisted of animals receiving serial dilutions of virus in the usual fashion (i.e., without saline injections). No significant differences were observed in the two types of controls. At all times, the number of animals was the same in the control groups as in the treated groups.

†In this experiment selected mice weighing 16 to 20 grams were used.

‡Two doses of drug daily for 5 days (8 mgms. in 1.0 ml. at 8 a.m. and another at 6 p.m.).

TABLE I (Continued)

Experiment	Results on	Total Dose of Drug in Milligrams	SURVIVORS/TOTAL in Dilutions of Virus Tested				% Survivors (14 Days)	Final Titer (14 Days)
			10^{-4}	10^{-5}	10^{-6}	10^{-7}		
D†	5th Day	80	10/15	14/15	12/15	13/15		
	14th		7/15	8/15	12/15	10/15	61	$10^{-5.0}$
	5th Day	40	8/15	11/15	11/15	14/15		
	14th		2/15	8/15	11/15	10/15	51	$10^{-5.4}$
	5th Day	0 (controls)	0/15	0/15	2/15	5/15		
	14th		0/15	0/15	0/15	5/15	8	$10^{-7.0}$
E†	5th Day	80	4/15	7/15	12/15	12/15		
	14th		3/15	6/15	11/15	12/15	53	$10^{-5.8}$
	5th Day	0 (controls)	0/15	0/15	1/15	7/15		
	14th		0/15	0/15	0/15	5/15	8	$10^{-7.0}$
F‡	5th Day	80	5/10	8/10	10/10	10/10		
	14th		5/10	7/10	9/10	9/10	75	$10^{-4.8}$
	5th Day	0 (controls)	0/10	0/10	2/10	5/10		
	14th		0/10	0/10	0/10	4/10	10	$10^{-7.0}$

*Control animals received intraperitoneal injections of 0.85% saline comparable in regard to volume of fluid and schedule with mice under treatment. Additional controls consisted of animals receiving serial dilutions of virus in the usual fashion (i.e., without saline injections). No significant differences were observed in the two types of controls. At all times, the number of animals was the same in the control groups as in the treated groups.

†In this experiment selected mice weighing 16 to 20 grams were used.

‡Two doses of drug daily for 5 days (8 mgms. in 1.0 ml. at 8 a.m. and another at 6 p.m.).

be demonstrated in all the tissues of the mouse. It seemed, therefore, that any therapeutic results obtained after a 24-hour infection period would be significant.

A summary of several experiments carried out under the experimental conditions stated above is given in Table I.

It is apparent from Table I that sodium phenosulfazole is effective against as many as 100 LD_{50}'s of the mouse poliomyelitis strain used in this study. It is of interest to note that no control mice survived in dilutions below $2x10^{-7}$, and since the lethality of this mouse strain has been frequently observed in this and other laboratories, the presence of survivors in the treated groups is of particular significance.

On the basis of these and other experiments, a strong impression has been received that with slight changes in the treatment schedule and careful selection of mice (18 to 20 grams) the compound under discussion is consistently effective against *at least* 100 intraperitoneal LD_{50}'s of SK mouse poliomyelitis.

Prophylactic Feeding

On two occasions sodium phenosulfazole has been given in 4 mgm. amounts to mice by gavage. Six to seven hours after the drug was placed in the stomach of the animals, they were injected intraperitoneally with 0.06 ml. of a 10^{-6} dilution of mouse brain pool. In the first experiment, 25 of 45 mice survived and in the second, 18 of 34 animals survived. In both experiments no control mouse survived the 10^{-6} assault dose of virus.

The survivors of the drug-treated animals were immune to a second dose of virus (10^{-6}) given intraperitoneally 3 weeks after the first dose.

This finding is considered one of the most important of the current study and is being investigated further with large numbers of animals.

In monkeys, drug dosage has ranged from 250 mgms. to 400 mgms. per kg. body weight. The results of these therapeutic studies will be published at a later date. For present purposes it is sufficient to note that the monkey schedule has been extended for as long as 10 days without apparent ill effect. On

one occasion post-mortems on 11 monkeys which had received 400 mgms. per kg. body weight for 10 days revealed, grossly, a complete lack of pathology.

On the other hand, it should be noted that our observations have been limited to mice and monkeys. If other species of animals are exposed to this drug, it will be necessary to determine the range of tolerance for each species. That sodium phenosulfazole is a powerful tissue reactor is apparent from our tissue culture experiments and from the reaction of one monkey studied for acute toxicity. This animal received a total of 1.0 gm. per kg. of a 25% suspension of drug administered intravenously during a 30-minute interval. Within 60 minutes following the injections the monkey showed signs of cortical stimulation, had convulsions and died. In contrast to this experience, a group of 3 monkeys received 2.0 gms. per kg. body weight over a period of 3 days and showed no ill effects.

Immunity in Treated Mice

It is worthy of note that evidence is available showing that mice infected with SK mouse poliomyelitis and successfully treated with sodium phenosulfazole are immune to reinfection with the same virus.

Monkey Experiments

The effect of sodium phenosulfazole is being studied in macacus rhesus monkeys infected by the intracerebral route with a human strain of poliomyelitis. This work is being extended to include various species of monkeys and routes of infection and will be reported in detail at a later date.

Tissue Culture Experiments

When in the early phases of this study phenosulfazole showed promise of exerting an antiviral effect in infected mice, it became of interest to determine whether the drug was acting directly on the virus or on the cellular substrate. Fluid tissue cultures were selected for this phase of the investigation since the mouse virus had been comprehensively studied *in vitro* and data were available for normal growth and deterioration curves.

The fluid cultures of serum ultrafiltrate and embryonic mouse brain were prepared in the same manner as previously described. To date, thirteen major experiments have been carried out with fluid cultures and phenosulfazole, and a detailed description will be included in a more comprehensive report. At this time it is desired to give a brief statement of our experiences emphasizing the factors which appear to be important in this type of work. The experiment summarized in Table II has been selected as representative.

When the virus was placed in a cell-free serum ultrafiltrate medium, the disappearance of the infectious agent followed the normal deterioration curve. Since it was soon apparent that phenosulfazole did not act directly on the virus, the drug was placed in infected embryonic mouse brain cultures to see what the effect would be on the virus growth curve. As may be seen in Table II, the inhibition of virus propagation was abrupt; the routine infected tissue culture having a mouse intracerebral titer of $10^{-5.5}$ and a parallel or sister culture to which 10 mgms. of phenosulfazole had been added having a titer of $10^{-1.0}$. This effect has been consistently obtained.

Because phenosulfazole is a sulfonamide, it was obviously desirable to see whether a parabenzoic compound would have an antagonistic effect on it. Accordingly, parahydroxybenzoic acid in 10 mgm. amounts was added to the culture-virus-phenosulfazole system. It is of interest that the antagonistic

TABLE II

The Effect of Phenosulfazole and Parahydroxybenzoic Acid on Virus Infected Tissue Cultures

Culture	Days incubated before titration in mice	Mouse Titer (intracerebral inoculation)
A. Forty-first serial passage of mouse virus in serum ultrafiltrate, embryonic mouse brain fluid cultures.	3	$10^{-5.5}$
B. 10 mgms. of Phenosulfazole added to Culture "A" (at time Culture "A" was titrated).	3	$10^{-1.3}$
C. 10 mgms. of Parahydroxybenzoic Acid added to Culture "B" (at time Culture "B" was titrated).	2 3 4	$10^{-2.5}$ $10^{-3.0}$ $10^{-3.3}$

effect was easily demonstrable. In the very same tissue culture (the one containing phenosulfazole in which the titer was $10^{-1.0}$) the addition of the parahydroxybenzoic acid produced an increase in titer of $10^{-3.3}$ in 96 hours.

The question may well be raised why the final virus potency, following manipulation of the culture with the drug and its antagonist, was lower than the original culture, *i.e.*, $10^{-3.3}$ as against $10^{-5.5}$. It must be remembered that the embryonic mouse brain cells which constitute the cellular elements of the fluid culture are, under optimal circumstances, in a quiescent or resting phase rather than in a proliferative phase. Such cells are susceptible to environmental changes and deteriorate rapidly. One of the most likely sources of trauma is the addition of the parahydroxybenzoic acid. It is apparent from the indicator present in the serum ultrafiltrate medium that an extreme change in pH takes place unless the chemical is added carefully.

Furthermore, the effect of ageing on the culture cannot be overlooked. By the time the parahydroxybenzoic acid phase of the experiment is tested 9 days have elapsed; 3 days for the routine growth of the virus in a freshly made culture; 3 days for the phenosulfazole, or inhibition phase; and 3 days for the neutralization phase of parahydroxybenzoic acid. If 9-day points are checked on the growth curve for this virus, it will be seen that $10^{-3.0}$ is an average value for routinely infected cultures. This fact was, of course, also checked with routine control cultures. It may be stated that the antagonistic action of parahydroxybenzoic acid is a complete one and that the lower final titers suggest a minimum of tissue damage. These observations are stressed because the phenosulfazole-parahydroxybenzoic acid reaction appears to be a promising instrument for the study of cell-virus relationships.

The various steps of the tissue cultures have occasionally been checked in plasma drop preparations to see what cellular damage occurred as the result of exposure of the tissues to the drug and its antagonist. It has been difficult to evaluate such preparations since normal embryonic mouse brain tissue removed from fluid tissue cultures to plasma cultures in Carrel flasks do not show much capacity for proliferation, although such cells still support virus growth. The correlation between

plasma preparations and fluid virus cultures is very important. This phase of the investigation is being studied.

Toxicity

One of the most striking aspects of the current study has been the apparent lack of toxicity of sodium phenosulfazole for mice and monkeys. Detailed data on the work done at the Lederle Laboratories and at Columbia University will be published in the near future. In our hands, the principle of non-toxicity was confirmed since it was not unusual to have large numbers of mice receive 1.0 gram of drug per kg. body weight for five days (Exps. B, D, Table I) for a total intraperitoneal dosage of 5.0 grams per kg. At no time were ill effects noted in these animals which were frequently observed for 21 days and longer. Numbers of these animals have been completely autopsied for microscopic studies, and there is evidence for believing that the gross impression is confirmed by tissue sections.

Conclusions

1. Sodium phenosulfazole (Darvisul), N-(2-thiazolyl)-phenol sulfonamide, is effective against as many as 100 LD_{50}'s of a mouse poliomyelitis virus when intraperitoneal treatment is instituted 24 hours after intraperitoneal infection.
2. The efficacy of this drug depends upon a proper dosage and time schedule.
3. The size of the mouse (16 to 20 grams) is important in carrying out the therapeutic studies.
4. Mice receiving treatment and surviving an initial infection are relatively immune to reinfection.
5. In two experiments more than half of the mice receiving a single dose of sodium phenosulfazole by mouth were resistant to a 10^{-6} virus challenge which killed all control animals. The survivors were immune.
6. Phenosulfazole in serum ultrafiltrate tissue cultures does not act directly on the mouse virus but appears to derive its antiviral properties by reacting with the cellular substrate.
7. Parahydroxybenzoic acid nullifies the antiviral effect of phenosulfazole in tissue cultures.

8. Sodium phenosulfazole has been strikingly non-toxic in the two experimental hosts studied, mice and monkeys.
9. The effect of sodium phenosulfazole is being studied in monkeys infected with a human strain of poliomyelitis.

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NOTE: Since receipt of this report, Doctor SubbaRow died suddenly of coronary disease. His death deprives his colleagues of a vigorous and inspiring associate.

Yellapragada SubbaRow was born in 1896 in Madras, where he received his collegiate training. As a Rockefeller Foundation Fellow, he obtained his doctoral degree from Harvard, where later he was associate professor of biochemistry. Since 1942 he has been Director of Research of the Lederle Laboratories. His chief interests were in phosphorus metabolism, liver factors, antibiotics, and bacterial nutrition.