ment, the inhibition of hemagglutination *in vitro* suggests a blocking action on cell receptors rather than any form of direct interaction between Col-SK virus and RDE principle. This concept is further supported by the initial presence of live virus at the portal of entry of the infected animal which has received RDE by the same route, as well as by the fact that a substantial degree of immunity to reinfection can be demonstrated in a majority of the surviving animals. It seems plausible that further experiments in which the cell-protective action of a potent enzymatic principle, such as RDE, is combined with the direct antiviral action of a colloidal dye of the Ro 2-3532 type, should permit the realization of even more powerful chemotherapeutic effects over a longer range of the infectious process.

Summary and Conclusions: The chemotherapeutic effect of a certain napthoquinonimine (compound Ro 2-3532/1) was studied on the peripheral infection of mice with Col-SK virus, F virus and EMC virus. It was found that a well tolerated single dose of 5 mg of the drug, administered intraperitoneally, was sufficient to protect a high percentage of the mice against infection with multiple paralytic doses of Col-SK or F virus injected simultaneously by the same route. Experiments in which the virus was injected intraperitoneally but the drug subcutaneously showed likewise a definite degree of protection though inferior to that observed when both were given by the same route. Significant protection could also be obtained up to and including 1 hour after intraperitoneal infection with small doses of virus. Experiments in which the drug was tested for its chemotherapeutic action against EMC virus showed only a minimum degree of protection considered to be insignificant. Some attempts were made to analyze the probable mode of action of the drug on Col-SK virus. It is concluded that protection occurs not because of cell-receptor blockade but that the virus is rendered non-infectious by some form of direct interaction between virus and drug.

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Chemotherapeutic Effect of 2-hydroxy-1,4-naphthoquinonimine on Infections of Mice with Col SK Virus. (18718)

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These studies were prompted by the observations of Woods and Rusoff(1) and Bieter and Wright(2) according to which the tetrazo dyestuff trypan red exerted a marked prophylactic effect on the intra-abdominal infection of mice with MM virus. These findings could not be reproduced in our experiments thus confirming the work of Hammon, Aird and Sather(3). Nevertheless, experimental work with various azo dyestuffs was included in the screening program with Col SK and MM virus which has been carried out in this laboratory. These azo dyestuffs, most of them derived from sulfonamides, also failed to show any protective effect on the infection with the above viruses. It was, however, observed that compounds of a series of 2-hydroxy-1,4-naphthoquinonimines possessed more or less marked activity, if administered to mice previously infected with MM- or Col SK-virus. A group of 160 compounds of this type has been tested during recent years and 67 members of the series were found to be active in the viral infection. In the present note we shall limit ourselves to the description of the chemotherapeutic property of one representa-

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2. Bieter, R. N., and Wright, H. N., *Poliomyelitis* 1949, p. 276. (J. B. Lippincott Co., Philadelphia)

3. Hammon, W. McD., Aird, R. B., and Sather, G., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 511.

tive compound which illustrates particularly well the general type of activity encountered in this group of compounds.

The compound is 3-(1,4-dihydro-3-hydroxy-4-oxonaphthylideneamino)-N-[β-(β-hydroxyethylamino)-ethyl]benzamide (Ro 2-3532), a substance of orange red color (U. S. Pat. 2,551,647). The compound was tested as the water soluble bisulfite derivative (Ro 2-3532/1) primarily because it was more convenient in experiments with small laboratory animals to use solutions rather than suspensions of the insoluble product (Ro 2-3532). The latter, however, was found to possess a similar type of basic activity.

Materials and methods. All experiments were carried out in young (16-18 days) mice of 10-12 g weight drawn from one colony. Animals from 2 other breeders were occasionally tested and found to respond equally well to the infection and the therapeutic treatment.

Strain of Col SK-virus. The strain of Col SK-virus was kindly given to us by Dr. C. W. Jungeblut, Department of Bacteriology, College of Physicians and Surgeons, Columbia University. The strain was passed through mice by intracerebral infection and for these experiments pools of infected brains were prepared and kept in the frozen state. Each viral pool consisted of the brains of about 60 mice infected intracerebrally 2 days before. Mice with the symptoms of flaccid paralysis were sacrificed, the brains harvested, weighed and emulsified with saline in a Waring blendor to give a 1-5 suspension. The suspension was then dispensed in 1.0 ml amounts into sterile rubber capped vials, quickly frozen in a dry ice-acetone mixture and stored at −10°C. For the experiments the contents of one ampul were thawed, and the desired virus dilutions prepared with saline. Since at least 2 and frequently 3 experiments were carried out every week, the pools were generally not used for longer than 8 weeks. The virulence of the different pools of infective brain was consistent and did not change appreciably during the comparatively short time of storage. An inoculum of 0.1 ml 10^{-6} or $10^{-5.7}$ was used in the majority of the experiments. These amounts were fatal for 90-100% of the infected animals and represent 10 to 20 50% lethal doses. The intra-abdominal infection of mice with 0.1 ml 10^{-7} dilution resulted in about 50% fatalities (*e.g.* 68 mice out of 125 = 54.5%) while 0.1 ml of a 10^{-8} dilution of brain suspension was fatal for only 12% of the animals.

Compound Ro 2-3532/1. The compound was first tested as a stabilized solution, but later on freshly prepared solutions were used. The substance dissolves readily with orange red color in distilled water; since the solutions are not stable, they were prepared shortly before use and not kept for longer than one to two hours. Dilutions in distilled water were prepared to contain the desired amount of drug in 0.5 ml.

Experimental procedure. In the standard experiments, groups of 10-20 mice were infected intra-abdominally with 0.1 ml of 10^{-6} or $10^{-5.7}$ diluted brain suspension, corresponding to 10-20 LD_{50}. During the time of the inoculation the tubes containing the dilutions of brain suspensions were kept in ice water. Immediately following the infection, the mice received a *single* intra-abdominal or subcutaneous injection of the drug in 0.5 ml per 10 g mouse. Single administration of the compound was used since numerous experiments with members of this class of compounds had demonstrated that repeated treatment with these substances, either on the same day or on consecutive days, did not result in more marked activity. Groups of untreated mice remained as controls after experience had shown that the injection of saline had no influence on the course of the infection. Titration of the virulence of the virus by including groups of mice with lower infective doses was carried out from time to time but was not routinely added to all experiments. Treated and untreated mice were observed to their death or, in case of survival, for a 21-day period, and symptoms of the infection were daily recorded. As a rule the first symptoms appeared 3 to 4 days after the infection and death occurred within the first 10 days. After that time no essential changes were observed in the experiments. Modifications of the site of the infection, as well as variations in the dose of the drug and its route of administra-

TABLE I. Effect of a Single Intra-abdominal Administration of Ro 2-3532/1 Immediately after the Intra-abdominal Infection of Mice with Col SK Virus.

Viral pool No.*	Virus LD_{50}	Drug dose mg/kg	No. of tests	Treated mice No.	Treated mice survivors No.	Treated mice survivors %	Control mice No.	Control mice survivors No.	Control mice survivors %	P =
6	10-20†	250	6	59	32	54.2	60	4	6.7	<.001
7	,,	250	7	69	52	75.5	70	2	2.8	<.001
3	,,	500	17	209	179	85.6	220	15	6.8	<.001
4	,,	500	23	387	308	79.6	418	5	1.2	<.001
5	,,	500	24	300	265	88.3	320	18	5.6	<.001
7	,,	500	17	222	160	72.0	205	12	5.8	<.001
5	100‡	500	2	20	2	10.0	20	0	0.0	ns§
6	,,	500	3	30	11	36.7	30	1	3.3	<.01
7	,,	500	3	40	1	2.5	30	0	0.0	ns

* Pools 3, 4, 5, 6, 7 were prepared on 6-1, 8-4, 9-22, 11-9 and 12-13-50 respectively and used to 8-8, 9-22, 10-30, 12-18-50 and 2-22-51 respectively.

† Dilution 10^{-6} or $10^{-5.7}$ of the virus suspension.

‡ Dilution 10^{-5} of the virus suspension.

§ ns = not significant.

TABLE II. Synopsis of the Data on Table I.

Virus LD_{50}	Drug dose mg/kg	No. of tests	Treated mice No.	Treated mice survivors No.	Treated mice survivors %	Control mice No.	Control mice survivors No.	Control mice survivors %	P =
10-20	250	13	128	84	65.6	130	6	4.6	<.001
10-20	500	81	1118	912	81.6	1163	50	4.4	<.001
100	500	8	90	14	15.6	80	1	1.3	<.01

tion will be mentioned in the experimental part.

Experimental. Toxicity. The toxic effect of a single injection of the compound Ro 2-3532/1 was determined in mice and rabbits. The LD_{50} for adult white mice of 16-20 g was 1070 mg/kg for the subcutaneous route, 818 mg/kg for intra-abdominal, and 422 mg/kg for intravenous injection. The young mice as used in the viral experiments were not essentially more sensitive; the intra-abdominal or subcutaneous injection of 500 mg/kg which was well tolerated by the older animals was also harmless for the younger mice. The LD_{50} for albino rabbits of 1.8-2.0 kg was found to be 168 mg/kg by the intravenous route. The toxicity of Ro 2-3532/1 might have been influenced by the presence of an excess of bisulfite in the comparatively high doses used. That sodium bisulfite can exert marked toxic effects has recently been shown by Hoppe and Goble(4). The insoluble naphthoquinonimine given as a suspension in gum arabic was less toxic. Amounts larger than 5.0 g/kg were tolerated *per os,* while the LD_{50} of an intra-abdominal injection was 1674 mg/kg, *i.e.* more than double the value found with the soluble derivative.

Experiments with Col SK-virus. In Table I a survey of the results of all experiments with the various viral pools is given and the totals are summarized in Table II. It is evident from these figures that with an infective dose of 10-20 LD_{50} which was fatal for an average of 95% of the untreated controls a significantly high survival rate was observed, particularly with the dose of 500 mg/kg. The variation of the percentage of survivors in the individual experiments was in no instance higher than 20%, indicating that the range of activity in the different experiments varied from an occasional minimum of 50% to a maximum of 90-100%. The lower treatment dose of 250 mg/kg was occasionally (*e.g.* with pool 6) of lower activity. Mice infected with higher virus doses, *e.g.* with 10^{-5} diluted brain suspension corresponding to 100 LD_{50}, did not respond satisfactorily to the treatment with the compound, although in one group of experiments a significant survival rate was found.

The doses of Ro 2-3532/1 used in these

4. Hoppe, J. O., and Goble, F. C., *J. Pharmacol.,* 1951, v101, 101.

TABLE III. Evaluation of the PD_{50}† of Ro 2-3532/1 in the Intra-abdominal Infection with Col SK-virus (10 LD_{50}).
Infection: 0.1 ml 10^{-6} brain suspension. Treatment: single intra-abdominal injection.

Dose, mg/kg	Survival ratio*	% survivors	PD_{50}, mg/kg†
10.0-12.5	11/40	27.5	
25.0	26/70	37.1	
50.0	39/70	55.7	44.4
100.0	44/70	62.9	
250.0	53/59	89.8	
500.0	30/30	100.0	
Controls	1/50	2.0	

* No. surviving/No. infected mice.
† 50% protective dose, calculated according to Reed and Muench.

experiments were multiples of the minimal active dose which was evaluated in a group of 4 experiments with almost identical results. The data were, therefore, combined in Table III. This table shows that as little as one-tenth and one twentieth of the well tolerated dose of 500 mg/kg exerted a marked effect if given immediately after the infection and at the same site. Significant survival rates ($p = <0.001$) were still observed with a dose of 25 mg/kg. Smaller doses of 10-12.5 mg/kg were no longer significantly active.

Two questions arose from the observations described so-far: Is the effect of Ro 2-3532/1 influenced by (1) the time of treatment and (2) by the site of the drug administration? From the experiment given in Table IV it is seen that a considerable drop of activity occurred, if a single dose of Ro 2-3532/1, 500 mg/kg was administered after a 15 to 60 minute interval between infection and treatment. After 90 minutes and later no convincing effect was seen at all. Although it is evident, that the course of the infection was noticeably changed in the treated animals and the death of the unsuccessfully treated animals was delayed, the survival rate was, if at all, only on the borderline of significance. Attempts to protect mice by giving Ro 2-3532/1 at different intervals before the infection have failed. The dose of 500 mg/kg which fully protected the animals if given simultaneously failed to show any effect even one hour before the infection.

On the other hand, it was possible to control an intra-abdominal infection by treatment with Ro 2-3532/1 remote from the site of the infection. Table V contains the results of a series of experiments in which a single large dose (500 mg/kg) of the compound was given subcutaneously following the intra-abdominal infection with 10-20 LD_{50} of the virus. Significant survival rates of an average of about 50% were observed in all groups of experiments. It was interesting to note that this property of Ro 2-3532/1 was not very common in the class of naphthoquinonimines. Even chemically closely related substances which exerted approximately the same activity as Ro 2-3532/1 when given intra-abdominally, *i.e.* at the site of the viral infection, failed to show an effect by the subcutaneous route. No explanation for the exceptional finding with Ro 2-3532/1 can be offered at the present time.

Practically the same results were obtained in experiments with MM virus: In 8 tests using an intra-abdominal infection of 10 LD_{50} 73 out of 79 mice survived after intra-abdominal treatment with 500 mg/kg ($= 92\%$), 40 out of 80 mice survived after the subcutaneous administration of the same dose ($= 50\%$) while 10 out of 122 untreated control animals remained alive (8.4%). Of other viruses only influenza virus type A (PR8-strain) was tested. Repeated subcutaneous treatment with Ro 2-3532/1 was without effect in the intra-nasal infection of mice.

According to our present experience the effect of Ro 2-3532/1 and the related compounds is limited to the intra-abdominal infection of mice with Col SK or MM virus. All attempts to influence intracerebral, intramuscular, subcutaneous and plantar infections with these viruses have failed so far. This seems to indicate that when virus is introduced in the peritoneal cavity, conditions are favorable to permit a sufficient exposure of the virus to active concentrations of the compound thus preventing infection of the central nervous systems in the majority of animals.

Very little information is at present available as to the possible mechanisms of activity of the naphthoquinonimines in the Col SK or MM infection. There is no indisputable evidence that Ro 2-3532/1 and related com-

TABLE IV. Effect of a Single Intra-abdominal Injection of Ro 2-3532/1 (500 mg/kg) at Different Intervals after the Intra-abdominal Infection with 10 LD_{50} of Col SK-virus.

Interval,* min.	No. of mice	No. of survivors after.....days							% survivors†	P =
		3	4	5	6	7	8	9		
0	30	27	26	26	26	26	26	26	90	<.001
15	30	28	17	13	13	13	12	11	36	<.05 >.02
30	30	29	26	21	19	18	15	15	50	<.01
60	30	26	22	17	14	12	12	12	40	<.02 >.01
90-120	40	36	28	12	9	9	9	9	23	n.s.‡
180-240	20	15	5	2	1	0			0	
Controls	30	27	16	6	5	4	3	3	10	

* Between infection and treatment.
† After 21 days.
‡ n.s. = not significant.

TABLE V. Effect of a Single Subcutaneous Administration of Ro 2-3532/1 on the Intra-abdominal Infection of Mice with Col SK-virus.
Infection: 10-20 LD_{50}*. Treatment: 500 mg/kg, immediately after infection.

Pool No.	No. of tests	Treated mice survivors			Control mice survivors			P =
		No.	No.	%	No.	No.	%	
3	1	20	12	60.0	20	2	10.0	<.01
5	5	60	37	61.7	60	2	3.3	<.001
7	6	90	40	44.5	70	2	2.9	<.001
Totals	12	170	89	52.3	150	6	4.0	<.001

* 10^{-6} dilution.

pounds exert an effect on the virus *in vitro*. Experiments with direct exposure of the virus to Ro 2-3532/1 *in vitro* were carried out by suspending infective brain in a final dilution of 10^{-2} in 0.5% to 2.5% solutions of the substance. These mixtures were kept at room temperature for 2 hours. The inoculum was then freed of the drug by washing and dilutions of 10^{-3}, 10^{-4}, 10^{-5} were prepared and injected intra-abdominally into groups of 5 mice for every dilution. A viral brain suspension in saline was treated in an identical manner and mice were infected with this material. In 2 out of 5 experiments all mice infected with the 10^{-5} dilution of the virus exposed to Ro 2-3532/1 survived. This occurred without relation to the drug concentration, *i.e.* in one instance after exposure to the 2.5%, in the other after exposure to the 0.5% solution of the substance. In the remaining 3 experiments all infected mice died like the controls. These findings seemed to suggest that, if at all, only a slight effect took place *in vitro*.

If unwashed mixtures of infective brain suspensions in concentrations ranging from 10^{-2} to 10^{-5} were prepared in 1% solution of Ro 2-3532/1 and injected into groups of 10 mice after 2 hours at room temperature (24°C), a survival rate of 70 to 100% was observed only with the 10^{-5} virus dilution. This represents a slightly greater anti-viral activity than is observed, if corresponding amounts of the compound and the virus were injected separately at the same site. However, the same result was obtained, if the mixture was injected immediately, indicating that prolonged exposure had failed to produce additional destruction of the virus.

Although the results *in vitro* were not entirely conclusive, there are, nevertheless, indications that Ro 2-3532/1 and related compounds possess direct anti-viral properties towards Col SK or MM virus at least *in vivo*. The most important is the fact that mice, surviving after administration of the active compound, could be successfully re-infected with a challenging dose (0.1 ml 10^{-5} dilution) of the virus 11-17 days after the infection. The reinfected animals succumbed to the infection at the same time as the controls.

Discussion. It is known that reproducible chemoprophylactic and chemotherapeutic effects in infections with small neurotropic viruses have only been rarely observed. We may add that 700 compounds from different chemical groups were tested in the Col SK and MM virus infections in this laboratory at the same time the naphthoquinonimines were studied and were found ineffective. Apart from the benzidine tetrazo dyestuffs(1) other substances such as arsenicals(5), certain sulfonamides(6), malononitrile(7) which had been described as active, were re-investigated

but like other investigators(8-13) we were unable to confirm these results. The interesting observations by Verlinde and de Baan(14) on the effect of *V. cholerae* filtrates in the infection of mice with SK and MM virus which was confirmed by Jungeblut(15) could also be reproduced by us. It appeared, however, doubtful whether the active principle of the crude filtrates was actually identical with Burnet's receptor destroying enzyme (RDE).

The observations with the naphthoquinonimine Ro 2-3532/1 as described in the experimental part suggest that this substance is able to control the infection of mice with Col SK virus with a marked degree of consistency. Recent work by Jungeblut(16) confirms and extends our findings. Although the effect of the new compound was limited to the *intra-abdominal infection* and could only be demonstrated with moderately high infective doses of the virus and short intervals between infection and treatment, the type of anti-viral activity suggested some form of direct interaction of the active substance and the virus. This might be inferred by the efficacy of a single administration, by the relation of the dosage to the effect and by the activity of the substance, if injected remote from the site of the infection. The experiments also seem to indicate that once virus has entered the cells of the host, it can no longer be influenced by the compound. Therefore, it might be assumed that the 'free' virus is prevented by the compound from reaching the cells in which it can multiply. This seems to be also borne out by the lack of immunity of the surviving animals.

In the absence of convincing evidence that the naphthoquinonimine exerted a direct activity on the virus *in vitro,* it may yet be assumed that one of the breakdown products of the large molecule which are formed by the organisms of the host might be the active principle *in vivo.* Further work on this problem is in progress.

Summary. (1) The group of 2-hydroxy-1,4-naphthoquinonimines contains compounds which show activity against infections with Col SK and MM virus in mice. The effect of one member of this class of compounds, designated Ro 2-3532/1 is described in detail. (2) A single intra-abdominal injection of the new compound (500 mg/kg), administered shortly after an intra-abdominal infection with 10-20 LD_{50} of Col SK virus prevented the death of approximately 80% of the animals. A single subcutaneous injection of the substance under similar experimental conditions protected about 50% of the animals. Surviving mice were fully susceptible to reinfection with Col SK virus. (3) The effect of the intra-abdominal administration of Ro 2-3532/1 was dependent on the dose of the compound, on the time of treatment and on the infective dose of the virus. (4) Successful treatment by either the intra-abdominal or subcutaneous route was only observed in intra-abdominally infected mice. Infections induced by intracerebral, intramuscular, subcutaneous or plantar injections of the virus did not respond to the treatment with Ro 2-3532/1.

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