In vivo Reversal by Cysteine of the Virustatic Effect of a Nitrofuran.* (19819)

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Previous observations in this laboratory have shown that 5-nitro-2-furaldehyde semicarbazones. or semioxamazones inhibit viruses of the psittacosis-lymphogranuloma group and the agent of primary atypical pneumonia in chick embryos and experimental animals (1,2). Recently Green and his collaborators have found the 5-nitro-2-furaldehyde semicarbazone (Furacin) acts as a limited sulfhydryl inhibitor reversible by cysteine, and that bacteria made resistant to Furacin show cross resistance to p-chloromercuribenzoate and trivalent arsenicals(3). Modifications to resistance appeared to be associated with changes in the SH groups of certain of the bacterial enzymes(4). Reduction of Furacin by tissues(5), bacteria, or cell free extracts is probably linked to a DPN-flavoprotein system(6,7) and occurs in the presence of a wide variety of oxidizable substrates. Cysteine accelerates the destruction of Furacin anaerobically in the presence of a partially purified xanthine-oxidase system from milk(8). This paper will report the reversal by cysteine of the inhibition of meningopneumonitis virus in the allantoic sac and present some experiments designed to indicate the site of reversal.

Materials and methods. Meningopneumonitis virus strain Cal 10 as used in these experiments had been carried through more than 30 yolk sac passages and 9 passages in the allantoic sac. Titrations of the ID_{50} were done in the allantoic sac and LD_{50} in the yolk sac. The compound subsequently referred to as NF 185 is 5-nitro-2-furaldehyde 4(diethylaminopropyl) semicarbazone. This drug because of its greater solubility was used in preference to Furacin. Solutions were sterilized by filtration through an ultra-fine Corning sintered glass filter. Cysteine hydrochloride solutions were sterilized by heat and after neutralization with NaOH were kept at 4°C under a vaseline seal. The degree of infection of the chick embryos was estimated by subinoculation of undiluted allantoic fluids into mice by the intranasal route and measuring the degree of pulmonary consolidation in terms of percentile lesion scores as previously described(1). NF 185 in the allantoic fluid was estimated by a modification of the spectrophotometric method described by Paul, *et al.*(9).

Effect of NF 185 on meningopneumonitis virus in embryonated eggs. To test the effect of NF 185 on the growth of meningopneumonitis virus in the allantoic sac of the chick embryo the following experimental setup has been adopted. The virus was inoculated into the allantoic sac in the amount of 100 I.D. contained in a volume of 0.1 cc. In order to avoid an immediate direct contact with the virus, the drug was inoculated into the yolk sac in a volume of 0.5 cc of solution. Both inoculations were performed within an hour. The age of the embryos used varied between 10 and 12 days. After inoculation the eggs were incubated for 5 days at 35°C and at the end of this period allantoic fluid from each egg was tested in 2 mice.

The results of these experiments are recorded in Table I. While all the control eggs at the end of the incubation period appeared heavily infected most of the eggs that received the drug did not contain enough virus to produce lung lesions in mice. Those found to be infected seemed to contain a much smaller amount of virus as compared with the controls. The data show that NF 185 has a chemotherapeutic effect against meningopneumonitis virus similar to that found for other nitrofurans(1) and that the minimal effective dose is between 0.5 and 1.0 mg under the conditions of these experiments.

Effect of NF 185 on meningopneumonitis virus in vitro. To establish whether the action of NF 185 on meningopneumonitis virus was

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TABLE I. Effect of NF 185 Injected into Yolk Sac on Growth of Meningopneumonitis Virus in Allantoic Sac.

Exp. No.	NF 185 in y.s., mg	No. of eggs containing detectable virus/total	Avg lesion score in mice†
1	2	*	
	1	1/5	2
	.5	3/4	24
	0 (controls)	5/5	76
2–5‡	1	4/16	5
	0 (controls)	21/21	87

* All the embryos died between the 1st and 3rd day after inoculation due to toxicity of the drug.

† Lesion score in %; avg for entire group of eggs. A lesion score of 100 represents death with complete pulmonary consolidation. In surviving mice 80 represents on the avg 4+ consolidation; 60, 3+; 40, 2+; and 20, 1+.

‡ Summary of results of 4 exp.

of virucidal or virustatic type, serial 10-fold dilutions of the virus were made in normal allantoic fluid and were incubated with NF 185 at concentrations of 200 γ/cc and 50 γ/cc including saline control for 6 hours at 35°C. At the end of the incubation 0.5 cc of the mixtures which originally contained 2,000, 200 and 20 LD_{50}/cc with each amount of drug were inoculated into the yolk sac of four 7-day-old embryos and the eggs incubated at 35°C for 12 days. The eggs were candled daily and the deaths recorded. There was no significant difference in mortality rate or time of death between the eggs inoculated with drug-virus mixtures and the controls with virus alone. The data demonstrate that NF 185, even in a concentration about 10 times that encountered in the allantoic fluid under our experimental conditions, does not seem to exert any appreciable virucidal effect *in vitro* on the meningopneumonitis virus.

Reversal by cysteine of the effect of NF 185 on virus growth. As an approach to the knowledge of the mechanism of action of NF 185 and also of chloramphenicol we tested two compounds, cysteine and p-hydroxybenzoic acid, as possible antagonists of antiviral activity. The inhibitors and virus were injected into the eggs as previously described. Cysteine or p-hydroxybenzoic acid was injected into the allantoic sac. In the experiments with cysteine this was given in daily doses of 10 mg and the treatment was started at different times after the inoculation of virus and nitrofuran. When the treatment with cysteine was begun at four days the eggs were incubated for 7 days instead of the usual time of 5 days. At the end of the incubation period the allantoic fluids were tested for presence of virus by intranasal inoculation in mice. The results in Table II clearly show that the injection of cysteine into the allantoic sac reverses the inhibition of virus growth caused by NF 185. It appears also that the reversal is possible as late as 96 hours after the inoculation of the virus and the drug. This finding suggests that the action of NF 185, as already observed in the experiments *in vitro,* is mainly of virustatic type. The virus is not killed by the nitrofuran but it remains in the allantoic sac for as long as four days without significant multiplication until cysteine is administered; then it promptly starts to increase. In eggs infected with meningopneumonitis virus and treated with minimal effective doses of 0.2 mg aureomycin or 1.0 mg of chloramphenicol, cysteine given as above did not have any effect on the inhibitory activity of these antibiotics.

Since the work of Davis(10) has shown that p-hydroxybenzoic acid is a growth factor for certain bacteria and that this substance reverses the bacteriostatic activity of p-nitrobenzoic acid, experiments similar to the above were undertaken both with NF 185 and with chloramphenicol. p-Hydroxybenzoic acid given either as a single dose of 10 mg or in daily doses of 5 mg for 5 days failed to diminish the virustatic activity of the 2 nitro compounds.

Evidence of the presence of NF 185 in the allantoic fluid after inoculation into the yolk sac. Eggs on the 10th day of incubation were inoculated into the yolk sac with 1 mg of NF 185 dissolved in 0.5 cc of saline. Another group of eggs of the same age was given 0.5 cc of saline only. The eggs were incubated at 35°C and at different intervals of time the allantoic fluids of 4 eggs of each group were harvested. If the allantoic fluid appeared grossly contaminated with blood it was discarded. The fluids were centrifuged for 15 minutes at 2,000 r.p.m., diluted 1:2 with saline and examined at the spectrophotometer

TABLE II. Reversal of Cysteine of Antiviral Effect of NF 185.

Cysteine, 10 mg daily, started at....hr	Eggs treated with Y.S.	Eggs treated with All.S.	Allantoic fluid tested for presence of virus at....days	No. of eggs containing detectable virus/total	Avg lesion score in mice
—	NF 185	S*	5	3/15	9
0	185	C	"	8/8	73
48	185	C	"	5/5	62
0	Saline	C	"	8/8	82
—	Saline	S	"	15/15	84
—	NF 185	S	7	3/11	9
72	185	C	"	5/5	82
96	185	C	"	9/9	70
—	Saline	S	"	12/12	81

* S = Saline; C = Cysteine.
Virus 100 ID_{50} in allantoic sac + NF 185 1 mg in yolk sac at time 0.

TABLE III. NF 185 in Allantoic Fluid at Different Intervals after Injection into Yolk Sac.

Hr after inj.	No. of eggs NF 185	No. of eggs Saline	Optical density of allantoic fluids at 380 mμ: NF 185 Range	NF 185 Avg	Saline Range	Saline Avg	Diff. avg	NF 185, γ/cc Min	NF 185, γ/cc Max	NF 185, γ/cc Avg
4	3	3	80- 90	85	70- 90	80				
8	3	3	145- 200	171	75- 85	80	91	3	6.5	4
16	3	3	290- 340	318	85- 95	90	228	8.4	10.4	9.6
24	4	4	430- 920	670	85-135	105	565	15.6	34.4	24
38	4	4	460- 670	545	95-200	147	398	13	22	17
48	4	4	410- 820	610	95-220	190	420	10	26.4	18
72	4	4	450-1100	681	165-265	217	464	10	37	20.6

in the region from 300 to 420 mμ; readings were taken at intervals of 10 mμ. A standard solution of NF 185 in saline containing 20 γ/cc was run in parallel with the allantoic fluids.

The first significant variation in the spectral absorption curve of the allantoic fluid from eggs that received the nitrofuran, as compared with the allantoic fluid of the control eggs, was observed 8 hours after the inoculation. The variation consisted in an increase of the optical density with its maximum at 380 mμ, corresponding to the peak of the absorption curve of NF 185. On the basis of the spectrophotometric analysis no evidence has been found for the appearance of other products possibly derived from the metabolism of the nitrofuran. The concentration of nitrofuran in the allantoic fluid was estimated by subtracting the average optical density of the allantoic fluids of the control eggs from the optical density of the allantoic fluids of eggs that were injected with nitrofuran. The concentration of nitrofuran was then established by comparing the values obtained with a standard titration curve. The results of these measurements are given in Table III.

Effect of cysteine injected into the allantoic sac on the nitrofuran content of allantoic fluid. A group of eggs that had received 1 mg NF 185 into the yolk sac 24 hours previously was given 10 mg of cysteine into the allantoic sac. Another group of eggs that had received the same amount of NF at the same time was given saline as control. The eggs were incubated at 35°C and at intervals of 6, 12 and 24 hours after the injection of cysteine the allantoic fluids from 3 eggs were examined for their NF content as previously described. A nitro-prusside test for the –SH group was also performed on each fluid.

In Table IV are recorded the results of this experiment. It will be seen that cysteine causes a temporary disappearance of the nitrofuran from the allantoic sac. The level is very low after 6 hours but by 12 hours it has returned to about half of the control value and at 24 hours there is little difference be-

TABLE IV. Analysis of Allantoic Fluid for NF 185 after Addition of Cysteine.

Hr after inj. of cysteine	NF 185 γ/cc in allantoic fluid from eggs given: Cysteine	Saline	Nitroprusside test*
6	3.5	24.6	+
12	12.4	19	—
24	20.4	22.8	—

* Nitroprusside test neg on eggs receiving saline.

tween the cysteine-treated and control groups. Assuming a concentration of cysteine in the allantoic fluid immediately after addition of 1 mg per cc the molar ratio of cysteine to NF 185 is of the order of 100:1. After the rapid disappearance of the cysteine the nitrofuran tends to reaccumulate in the allantoic sac.

Discussion. Cysteine added in the test tube to allantoic fluid containing NF 185 produces only a small reduction (about 10%) of the nitro-compound. The observed decrease of nitrofuran in the allantoic sac must be due, therefore, either to the activity of the lining cells of the sac or to enzymes in the allantoic fluid. The temporary reduction of the concentration of inhibitor in the fluid may result in a decreased amount within the cells thus permitting growth of virus. Since the nitrofuran soon reappears in the allantoic fluid it seems unlikely that there are important changes in the blood concentration.

Because of the lack of *in vitro* action of the nitrofuran at the experimental concentrations on meningopneumonitis virus it can reasonably be assumed that the important effects are produced in the cells lining the allantoic sac. It is not possible to tell on the basis of these experiments whether the inhibition of growth is due to an action on the virus itself or to disturbance of the metabolic activities of the host. If the cysteine acts intracellularly the observed reversal of inhibition could come about through dissociation of the nitrofuran from a sulfhydryl complex or by an increased rate of reduction of the nitrofuran to an inactive form. The experimental data indicate that the drug persists for long periods of time in the embryo after a single injection into the yolk sac. Reversal of its effect by cysteine as late as 96 hours after infection supports the concept of a prolonged virustatic activity in the tissues of the allantoic sac.

Summary. 1. The virustatic effect of 5-nitro-2-furaldehyde 4(diethylaminopropyl) semicarbazone on meningopneumonitis virus in the allantoic sac is reversed by cysteine given as late as 96 hours after the drug and virus. Injection of 10 mg of cysteine reduces temporarily the amount of nitrofuran in the allantoic fluid. 2. Since the nitrofuran had no *in vitro* virucidal effect at the concentrations used, the observed virustatic effect and its reversal are probably intracellular. 3. Cysteine did not reverse the virustatic effect of chloramphenicol or aureomycin.

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