THE GROWTH CURVE OF THE LANSING STRAIN OF POLIOMYELITIS VIRUS IN MICE: THE EFFECT OF SODIUM MONOFLUOROACETATE AND METHIONINE SULFOXIMINE ON THE EARLY PHASE OF GROWTH OF THE VIRUS*

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Recent studies from this laboratory have demonstrated that the administration of sodium fluoroacetate to mice infected intranasally with influenza virus inhibits the growth of that virus in the lungs of the mice (1). Fluoroacetate appears to exert its effect by interrupting the Krebs cycle, so that citric acid is not utilized in the lung, with a resultant disturbance of cellular function. The multiplication of influenza virus is inhibited in tissue culture by malonic acid and antimycin A, both substances which block the Krebs cycle by inhibiting succinic dehydrogenase (2). These findings emphasize the importance of this cycle to virus propagation.

Both DL-methoxime and DL-ethionine inhibit the growth of influenza virus in tissue culture (3). The effect of these compounds is prevented by methionine, of which they are analogues, suggesting that protein synthesis at this level, too, is important to virus growth. Under similar conditions another analogue of methionine, DL-methionine sulfoximine (4), which has been shown to be a reversible antagonist of methionine in several biological systems (5), also hinders the growth of influenza virus. Multiplication of the Lansing strain of poliomyelitis virus on tissue culture is also inhibited by DL-ethionine; the influence again is countered by methionine (6).

In these instances there is interference with basic metabolic activities of the cell which appear to be essential to the development of virus in the cell. The thesis has been accepted that investigation of substances, the influence of which upon fundamental cellular reactions is known, will contribute to understanding of the factors at play in the biosynthesis of different viruses. Since fluoroacetate has been shown to block the citric acid cycle in the brain of the rat (7) and in the central nervous system of the mouse (1), it was of interest to determine the influence of that compound upon the growth of the Lansing strain of poliomyelitis virus in the brain and cord of the mouse. The effect of methionine sulfoximine was examined as well. Preliminary studies revealed

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that in mice treated with these materials there was a delay in the onset of signs of disease, although the ultimate morbidity and mortality was usually the same as in untreated animals. Attention was then turned to the more quantitative procedure of measuring the influence of the chemicals upon the growth curve of virus in the nervous tissues as described in the preceding paper. The present report deals with the results of those studies (8).

Materials and Methods

Virus.—A pool of the 85th mouse passage of the Lansing strain of poliomyelitis virus was used. It had an average titer of $10^{-4.4}$ LD_{50} (50 per cent lethal dose). Ten to 20 per cent suspensions in sterile saline (0.15 M NaCl) were made by grinding tissues in a mortar with alundum. Intracerebral inoculation consisted in the injection of 0.03 ml. of the desired concentration of inoculum.

Mice.—White Swiss mice, 3 to 8 weeks old and 11 to 18 gm. in weight were used. Mice of approximately the same weight were used in each experiment. They were observed at least once a day, those which died within 24 hours were excluded from the calculations, and tests were concluded on the 30th day after intracerebral inoculation. Since the onset of paralysis in mice receiving this strain of virus is almost uniformly followed within 24 hours by death, morbidity and mortality are much the same but the time is different. The method of deriving cumulative morbidity has been described (8).

Subinoculation.—For the growth curves brains and cords were harvested from 5 or 10 mice at appropriate intervals after intracerebral inoculation, according to the procedure previously described (1). Brains were weighed separately from the cords (which in all instances included the medullae), stored at −70° C. until ground to make 10 per cent or 20 per cent suspensions which were stored at 4° C. for 1 to 10 days before subinoculation into 8 mice. All suspensions from control and treated mice obtained at one time after inoculation were titrated simultaneously by the intracerebral route. Subinoculation was carried out with 3 to 6 consecutive 10-fold dilutions between 10^{-1} and 10^{-6}. Titers are expressed as the LD_{50} and calculated according to the method of Reed and Muench (9).

Sodium Monofluoroacetate.—The preparation used was free of fluoride ions and was prepared by Monsanto Chemical Company. Solutions were prepared in sterile saline immediately prior to use, and 0.1 ml. was injected into each mouse by the intraperitoneal route.

DL-*Methionine Sulfoximine.*—The preparation used (P76-250-201) was supplied through the courtesy of Dr. L. Reiner of the Wallace and Tiernan Products, Inc. Solutions were prepared in sterile distilled water immediately prior to use, and 0.1 ml. was injected into each mouse by the intraperitoneal route.

EXPERIMENTAL

1. The Effect of Sodium Monofluoroacetate on Infection with Poliomyelitis Virus in Mice

Effect on Growth Curve of Virus.—In three experiments mice were injected intraperitoneally with 80 gamma of sodium monofluoroacetate in 0.1 ml. of saline, an average dosage of 6 mg./kg. of body weight. In the second experiment, one group of mice received 3 mg./kg. The drug was injected 1 hour before the intracerebral inoculation of 10 per cent suspension of virus (approximately 2000 LD_{50}); 1 per cent virus was used in one set of mice in the third experiment.

The results of pretreatment with a single injection of fluoroacetate on the growth of poliomyelitis virus are presented in Table I, and a typical experiment is presented in Fig. 1.

The curve of virus multiplication in the brain and spinal cords of untreated mice follows a regular pattern (8). After inoculation of 10 per cent suspension of virus the amount recoverable from the brain declines for about 6 hours to a level well below that of the original inoculum. Between 6 and 9 hours a sharp rise takes place and proceeds to a maximum in 24 to 30 hours after injection. In contrast, no evidence of multiplication of virus is observed in the brains of the treated mice up to 12 hours after the inoculation of virus so that titers

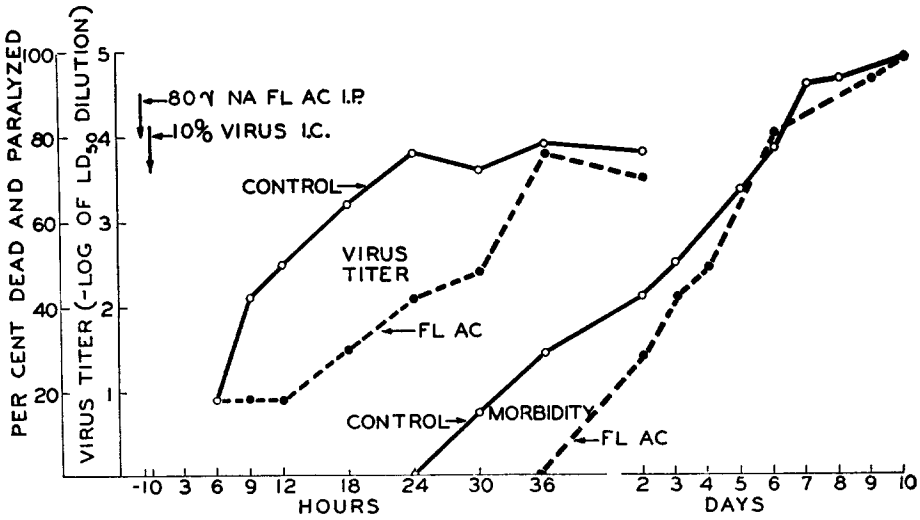

FIG. 1. Effect of sodium monofluoroacetate on the growth of virus in the brain and on the morbidity of mice inoculated with the Lansing strain of poliomyelitis virus.

remain at 0.9 and 0.6 (Experiments 2 and 3), while in the controls they have reached 2.6 and 2.3 logs, respectively, a 50-fold difference. In 18 hours multiplication of virus in the treated mice has begun but with the further increase in titer in the controls the difference in virus content remains about the same as at 12 hours. The difference tends to persist up to 24 to 30 hours, indicating that in both sets of animals multiplication of virus is proceeding at the same rate. The maximal titer of virus in controls is reached in that time while multiplication in the treated animals continues until, at 36 hours, a level equivalent to that in the controls is attained. Thus the influence of fluoroacetate is essentially to suppress multiplication of virus in the brain for 6 to 12 hours after which growth of virus proceeds at the usual normal rate. The retardation in multiplication resembles the delay observed when the curve of growth in the

spinal cords of mice after inoculation of 1 per cent virus is compared with that found after a 10 per cent inoculum, suggesting that a proportion of the virus has been inactivated by exposure to the chemical (8). It is of interest that in the one series in which 6 mg./kg. of fluoroacetate and 1 per cent virus were used (Experiment 3) the rate of multiplication was retarded for a longer period than

TABLE I

Effect of Sodium Monofluoroacetate on the Growth Curve of the Lansing Strain of Poliomyelitis Virus in Mice

Experiment No.	Virus concentration	Drug‡	Organ	Titer of virus after inoculation in brain or cord*															
				Hours												Days			
				0	3	6	9	12	18	24	30	33	36	48	65	3	4	6	10
	per cent	*mg./kg.*																	
		0	Brain	2.2	1.6	0.9	2.7	3.2	3.5	4.4			4.4						
1	10		Cord	0.6	0.7	0.9	2.1	3.0	4.3	4.3			4.1						
		6	Brain				1.1			1.7			3.7						
			Cord				1.3			1.4			3.7						
		0	Brain			0.8	2.1	2.6	3.2	3.8	3.6		3.9	3.8	4.5			3.7	2.7
			Cord			0.5	1.8	2.3	4.4	2.6	4.5		3.6	4.5	3.4			4.6	2.9
2		3	Brain			0.9		2.8	2.6	3.2	4.1		3.8	3.8					
	10		Cord			1.1		2.7	2.8	3.3	4.0		4.3	4.8					
		6	Brain			0.9		0.9	1.5	2.1	2.3		3.8	3.5	5.2				
			Cord			1.1		1.2	1.5	2.0	2.0		5.0	3.7	4.0				
		0	Brain	1.4	1.0	0.6	2.3	2.4	3.1	3.9	4.1	3.5	3.5	4.0	3.6	3.6	3.7		
	10		Cord	0.6	0.6	0.6	2.8	2.3	2.5	4.2	5.4	3.6	3.8	3.5	2.7	3.6	3.4		
		6	Brain	1.0	0.9	0.6	0.6	0.6	1.5	3.5	3.5	3.5	3.3	3.7	3.8	3.1	3.5		
			Cord	0.8	0.7	0.6	0.5	0.5	1.3	2.2	1.5	3.9	3.3	2.7	2.7	2.6	2.7		
3																			
		0	Brain	0.6	0.7	0.5	1.5	1.8	2.5	3.5	3.8	4.1	3.6	3.9	3.9	3.2	3.5		
	1		Cord	0.5	0.5	0.6	0.6	0.8	1.6	4.0	4.3	3.5	4.0	3.1	2.6	2.7	2.6		
		6	Brain	0.6	0.8	0.6	0.5	1.0	1.7	1.5	1.9	2.5	2.7	3.3	3.3	3.4	2.9		
			Cord	0.6	0.6	0.6	0.5	0.6	0.9	2.2	3.5	1.6	3.0	2.6	2.6	2.5	3.5		

* Titers recorded as $-\log$ of LD_{50} dilution.

‡ Drug injected intraperitoneally 1 hour before the intracerebral inoculation of virus.

in mice receiving 10 per cent virus, and the level of virus in either the brain or cord of treated animals did not equal that in the controls within the first 3 days. This suggests a reduction in the amount of virus capable of propagation rather than a complete escape of all potential virus from the influence of the chemical. When, in Experiment 2, the amount of fluoroacetate was reduced to half, the effect was largely lost.

Effect on Morbidity.—It was pointed out (8) that signs of disease ordinarily

begin in mice 6 hours or more after the virus content of brain reaches its peak. The data presented in Fig. 1 and Table II again illustrate this fact. However, in animals treated with fluoroacetate there is a marked delay in the rate at which the disease develops so that between 24 and 48 hours after inoculation of virus the morbidity may be that noted in the controls. Thereafter, the number of affected mice progressively increases, so that by the 10th day all animals in both groups have succumbed. The delay in onset of signs of disease among the treated animals reflects the inhibition in the rate of multiplication of virus observed in the nervous tissue.

Toxicity in Mice.—The intraperitoneal administration of sodium fluoroacetate in a dosage of 6 mg./kg. 1 hour before the intracerebral inoculation of virus resulted in the death of 15 to 22 per cent of 365 mice within 5 hours,

TABLE II

The Effect of Sodium Monofluoroacetate on the Morbidity in Mice Infected with Poliomyelitis Virus

Experiment No.	Virus concentration	Drug	Cumulative per cent of mice paralyzed and dead at intervals after intracerebral inoculation of virus														
			Hours							Days							
			24	27	33	36	40	48	50	3	4	5	6	7	8	9	10	11
	per cent	*mg./kg.*																
1	10	0	1			30		42			64	73	77				100	
		6	0			5		34			48	63	63				100	
3	10	0		14	34		49		60	81	96		97			100		
		6		4	7		13		31	42	49		81			93	98	100

whereas, less than 0.5 per cent of the untreated control mice died within 24 hours of the intracerebral inoculation of virus. The other mice treated with drug recovered from the gross toxic effect so that they appeared normal within 24 hours after the inoculation of virus. In mice not inoculated intracerebrally the same dosage of fluoroacetate resulted in a mortality of approximately 10 per cent. The dose of 40 gamma resulted in a 3 per cent mortality in the mice of the second experiment, while controls not inoculated intracerebrally survived the 40 gamma dose with only transient signs of toxicity.

The Effect of Sodium Monofluoroacetate on Virus in Vitro.—Because certain of the data suggest that the effect of influenza virus may be exerted directly on virus in the tissues, it was important to test its capacity to inactivate the virus *in vitro*. Equal parts of fluoroacetate and 10-fold dilutions of virus were mixed to give final concentrations of 2000 gamma fluoroacetate per ml. The virus-drug mixtures and appropriate virus controls were incubated for 1 hour at 37°C. Each mixture was dialyzed for 18 hours through a cellophane mem-

brane against 1000 volumes of distilled water held at 4°C. A third set of virus dilutions was kept at 4°C. to serve as a control upon the effect of incubation and dialysis. One other virus control consisted of undiluted (20 per cent) virus suspension held for 19 hours at 4°C. and diluted immediately prior to titration. Inoculations for the four titrations were made at the same time. It can be seen that the fluoroacetate had no destructive effect upon even the lowest concentrations of poliomyelitis virus *in vitro* (Table III).

TABLE III

Effect of Sodium Monofluoroacetate on Lansing Virus in Vitro

Treatment		Titration					Titer
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	(LD_{50})
FlAc	2000 γ FlAc/ml., 1 hr. at 37° C.; dialyzed 18 hrs. at 4°C.	8/8	7/8	7/8	1/8	0/8	$10^{-3.4}$
Incubation and dialysis	1 hr.at 37°C.; dialyzed 18 hrs. at 4°C.	9/9	8/8	5/8	2/8	0/8	$10^{-3.4}$
Dilution and storage control	Stored at 4°C. for 19 hrs.	8/8	8/8	7/8	5/8	0/8	$10^{-4.1}$
Virus control	20 per cent suspension held at 4°C. for 19 hrs. diluted immediately prior to inoculation.	9/9	8/8	8/8	0/8	0/8	$10^{-3.5}$

8/8 indicates 8 deaths in 8 mice inoculated.

2. *The Effect of* DL-*Methionine Sulfoximine on Infection with Poliomyelitis Virus in Mice*

Mice were injected intraperitoneally with 2 mg. of DL-methionine sulfoximine (an average of 150 mg./kg.) in 0.1 ml. of distilled water; in one experiment other mice received 1 mg. One hour later the treated and control mice were inoculated intracerebrally with a 10 per cent suspension of the Lansing strain of virus (approximately 2000 LD_{50}). In these experiments 5 mice from each group were sacrificed at various times following inoculation of virus.

The results are presented in Table IV. The second experiment is illustrated in Fig. 2. In each instance injection of 150 mg./kg. of the chemical agent inhibited the early phase of virus growth. The influence was most prominent and persistent in the first experiment, was also observed in the second, but was less apparent in the third.

There is less definite evidence of influence upon the initiation of growth than with fluoroacetate although the data of Experiment 1 are strongly suggestive;

TABLE IV

Effect of Methionine Sulfoximine on the Growth Curve of the Lansing Strain of Poliomyelitis Virus in Mice

Experiment No.	Drug‡ before 10 per cent virus	Organ	Titer of virus after inoculation in brain or cord*										
			Hours										
			8	12	15	18	22	28	31	42	55	83	103
	mg./kg.												
1	0	Brain				3.3		4.2		4.3			
		Cord				2.5		5.2		4.8			
		Morbidity, *per cent*..				0		17		50			
	150	Brain				0.7		2.7		3.4			
		Cord				1.7		4.2		4.9			
		Morbidity, *per cent*..				0		0		29			
2	0	Brain	1.8	3.2	3.3		3.5		3.8	4.5	4.1		3.4
		Morbidity, *per cent*..					0		2	15	18	36	61
	150	Brain	1.1	1.5	1.8		2.5		3.6	4.5	3.5		3.8
		Morbidity, *per cent*..					0		0	5	16	36	45
3	0	Brain	1.3	2.4	2.8		3.4		3.3	4.2	3.9		4.0
		Morbidity, *per cent*..					0		2	14	17	38	55
	150	Brain	0.7	2.2	2.6		2.5		3.3	3.1	4.1		3.5
		Morbidity, *per cent*..					0		0	2	13	35	57
4	0	Morbidity, *per cent*..	0				2		16	25		41	
	75	Morbidity, *per cent*..	0				0		2	14		20	
	150	Morbidity, *per cent*..	0				0		2	34		50	

* Titers recorded as $-\log$ of LD_{50} dilution.

‡ Drug injected intraperitoneally 1 hour before the intracerebral inoculation of virus.

those of Experiment 2 are more in keeping with the idea that there is a general slowing up of virus reproduction.

The onset of signs of illness was slightly delayed in all four groups treated

with 150 mg./kg. and in the one group treated with 75 mg./kg. but the difference between them and the controls was small and of brief duration. The mice used in the second and third experiments were from a source which was different from that of the mice used in the other experiments. This may in part account for the longer incubation periods observed before the onset of paralysis in mice of the second and third experiments.

The toxicity for mice of 150 mg./kg. of methionine sulfoximine by intraperitoneal administration is high. Between 29 per cent and 34 per cent of the 280 treated mice died within 12 hours. No further deaths occurred in the treated groups until after the earliest appearance of paralysis in them, approximately

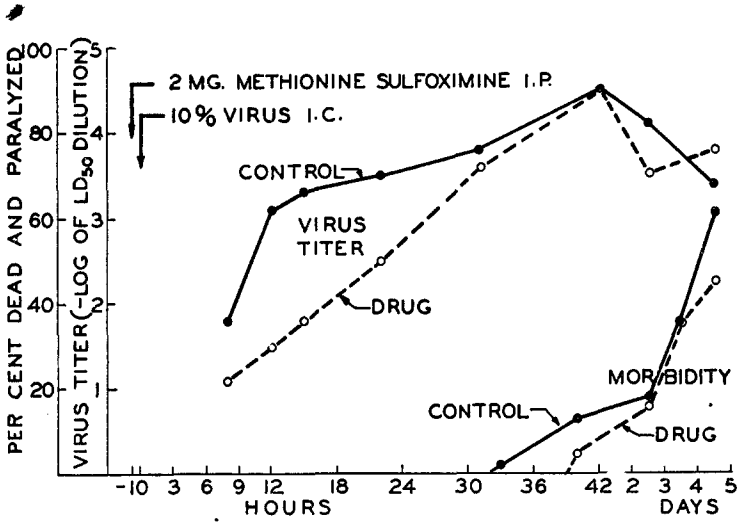

FIG. 2. Effect of methionine sulfoximine on the growth of virus in the brain and on the morbidity of mice inoculated with the Lansing strain of poliomyelitis virus.

40 hours. The mortality from drug was not seriously heightened by intracerebral inoculation. No deaths occurred within 24 hours after the injection of 75 mg./kg. into 50 mice 1 hour before the intracerebral inoculation of virus; transient signs of toxicity were seen.

DISCUSSION

The present results indicate that the metabolic processes taking place in the host, which are required for the synthesis of the neurotropic poliomyelitis virus are similar in at least two respects to those required for the synthesis of influenza virus. The growth of both viruses in animals is inhibited by the administration of sodium fluoroacetate (1), and by DL-methionine sulfoximine (4) and in tissue culture by ethionine (3, 6). Thus the interpretation of Ackermann (1) of the experiments with influenza virus is supported, namely, that the

proper functioning of the Krebs cycle is intimately related to the growth of virus (1, 2).

The present experiments, like those with influenza virus (1), demonstrate that fluoroacetate does not destroy the virus upon simple contact and the effect of both fluoroacetate and methionine sulfoximine appears only to modify the infection by delaying the onset of virus multiplication. The experiments do not indicate whether initial infection of the host cells is delayed until the effect or concentration of the drug is diminished, but practically all mice become fatally infected after a single injection of drug. Similar studies with multiple doses of the drug have not been completed.

A drug which alters the metabolism of the host cell may act on such fundamental processes as to block synthesis not only of virus but also of components essential to the cell. Or it could act on the synthesis of virus through later steps which might not be held in common with the host cell or which are not essential for the survival of the host cell. It is possible that the viruses of poliomyelitis and influenza require two identical or closely similar metabolic steps, which are blocked by fluoroacetate and methionine sulfoximine. That the reactions affected are of great importance in the normal host metabolism, can be deduced from the previously known action of fluoroacetate and methionine sulfoximine. Many reports on fluoroacetate are available which clearly demonstrate its capacity to alter the metabolism of the host cell (10), and especially to inhibit the oxidation of citric acid (1, 7). Similarly, it has been demonstrated in several biological systems that methionine sulfoximine is a reversible antagonist of methionine (5). Furthermore, the toxicity of the drugs, as seen in the present studies, indicates that they interfere with processes fundamental to the survival of the host. Whether the toxic effects are due to inhibition of processes necessary for both the survival of the host and the synthesis of virus in the infected tissue, or due to inhibition of essential metabolism at other sites in the cell or in other organs in the body, is not known at this time.

The essential contribution of this study would seem to be in calling attention to two of the possible sites in the metabolic processes of the host at which synthesis of the virus of poliomyelitis might be interrupted. Perhaps of greater significance is the demonstration that the formation of poliomyelitis virus in the infected animals, like that of influenza virus (1–4), is dependent upon processes which are an integral part of the metabolism of the cell.

SUMMARY

The early phase of growth of the Lansing strain of poliomyelitis virus in the mouse brain and cord was suppressed, or delayed, by the intraperitoneal administration of sodium monofluoroacetate in doses of 6 mg./kg. but not of 3 mg./kg. 1 hour before the intracerebral inoculation of virus. There was also a delay in the time at which the first signs of illness appeared in the treated mice.

Similar effects, but to a lesser degree, were observed after the administration of DL-methionine sulfoximine in doses of 150 mg./kg. and 75 mg./kg. Sodium fluoroacetate in very high concentration had no direct effect *in vitro* on the infectivity of poliomyelitis virus. It was found that in each instance in which the growth of virus was delayed, the time at which the first obvious signs of illness appeared was also delayed in a proportionate manner.

The significance of these findings is discussed, as well as the use of the growth curve of poliomyelitis virus in mice in studying the relation of cellular metabolism to viral synthesis and the search for effective chemical agents.

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