THE RELATION OF THE KREBS CYCLE TO VIRAL SYNTHESIS

II. The Effect of Sodium Fluoroacetate on the Propagation of Influenza Virus in Mice*

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It was concluded from previous investigations that certain chemical reactions involved in the synthesis of influenza virus are coupled with some oxidative reactions of the Krebs cycle (1). In the experimental system used, the propagation of influenza virus was found to be an aerobic process sensitive to the suppression of respiration of the host tissue by controlled oxygen tension or by chemical inhibitors of enzymes of the citric acid cycle. The interpretation of these findings is somewhat restricted by the limitations which are always associated with studies using excised tissues. Tentatively it may be suggested that the functioning of these enzyme systems in the citric acid cycle is for the production of energy necessary for the synthesis of viral protein. The inability of the host-virus system to derive this energy from glycolysis may be due to the absence of a mechanism whereby this energy-yielding process can be coupled to the viral synthetic reactions. It is also possible that the energy available from this source is quantitatively inadequate because of the innate biochemical nature of the host tissue or because of the conditions under which the tissue is surviving. It is conceivable that *in situ* the rate of glycolysis of a tissue with a blood supply may be quite different from that of the same tissue surviving in Simms solution. Thus in the intact animal the requirement by the virus for the enzyme systems of the Krebs cycle may or may not be specific. Hence, it has seemed desirable to investigate *in vivo* the relation of the Krebs cycle to viral synthesis.

The recent work of Buffa and Peters (2, 3), Potter and Busch (4), and Elliott and Kalnitsky (5) provided an effective tool for the furtherance of this study. By the use of sodium fluoroacetate, it is possible to inactivate the enzyme systems necessary for the oxidation of citric acid. Further, the actual time at which the blocking of this metabolic step is operative can be determined. Under the conditions of this alteration in metabolism, the propagation of influenza

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virus in the lungs of mice has been studied. The details and results of this investigation are reported below.

Materials and Methods

Virus.—The PR8 strain of Type A influenza virus was used exclusively in this study. Since its isolation from man, it has undergone 7 passages in ferrets, 593 passages in mice, and 127 passages in eggs.

Mice.—In all experiments Swiss white mice (Webster strain) were used. They weighed from 10 to 18 gm. and were matched by weight for each particular experiment.

Virus Titers.—The amount of virus in the lungs of mice was estimated by determining the infectious titer for eggs. For this purpose infected lungs were ground in a mortar with alundum and diluted with beef infusion broth to yield a 10 per cent suspension. Tenfold serial dilutions of this viral suspension were prepared and 4 eggs were inoculated with 0.1 ml. of each dilution. The 50 per cent infectious titer was calculated using the method of Reed and Muench (6). The virus titer of allantoic fluid was also estimated by red cell agglutination using a pattern method (7).

Citric Acid.—The citric acid content of lungs was determined by the pentabromoacetone method as modified by Natelson *et al.* (8). For this determination the tissue was prepared for analysis as described by Potter and Busch (4).

Sodium Monofluoroacetate.—The sodium monofluoroacetate used was prepared by Monsanto Chemical Co. and was free of fluoride ion. Solutions of suitable concentrations were prepared in saline and adjusted to a pH of 7.4. In all instances the sodium fluoroacetate solutions were prepared just prior to use and were administered intraperitoneally.

RESULTS

Effect of Sodium Fluoroacetate on Influenza Virus in Vitro.—

Various concentrations of sodium fluoroacetate were prepared in 0.15 M saline. These preparations were added to equal volumes of allantoic fluid containing influenza virus. Control preparations were made by mixing equal volumes of infected allantoic fluid and physiological salt solution. Some of the mixtures were incubated for 18 hours at 4° and others for 18 hours at 37°. At the end of the incubation period, the titers of infectivity in eggs and of hemagglutinin for chicken cells were determined. The maximum dose of fluoroacetate administered to mice in the studies reported here was 0.08 mg. per mouse.

Concentrations of sodium fluoroacetate as great as 2.0 mg. per ml. at 37° had no effect on the infectivity or hemagglutinin titer of the virus (Table I). Sodium fluoroacetate is not virucidal in that it does not destroy influenza virus on contact *in vitro*.

Effect of Sodium Fluoroacetate on the Citric Acid Content of Mouse Lung in Vivo.—

Mice were injected intraperitoneally with sodium fluoroacetate in saline at a dosage of 4 mg. per kg. of body weight. In subsequent experiments it will be demonstrated that this dosage will produce a marked effect on the propagation of influenza virus in the lung. At various intervals after the injection of fluoroacetate, groups of six mice were sacrificed. Citric acid determinations were made on pooled samples of the lungs.

The citric acid content of the lung had nearly doubled within ½ hour after the administration of fluoroacetate and by 1 hour it had tripled. The maximum increase detected (tenfold) had occurred by 8 hours and by 12 hours the citric

TABLE I

Effect of Sodium Fluoroacetate on Influenza Virus in Vitro

Temperature	Concentration* of NaFAc	Virus titer	
		Egg infectivity‡	Hemagglutination
°C.	γ/ml.		
4	000	8.3	1024
4	1000	8.7	1024
4	2000	8.7	1024
37	000	6.7	512
37	1000	6.7	512
37	2000	6.7	512

Equal volumes of sodium fluoroacetate (NaFAc) in saline were added to allantoic fluid containing influenza virus and incubated at different temperatures for 18 hours.

* Concentration recorded is the final value.

‡ Titer expressed as the log of the reciprocal of the dilution.

TABLE II

Effect of Sodium Fluoroacetate on the Citrate Content of the Mouse Lung

Time after NaFAc injection	Citric acid
hrs.	*γ/gm. of tissue*
0.0	78
0.5	140
1.0	224
2.0	525
4.0	617
8.0	885
12.0	322
24.0	292
36.0	113

Mice were injected intraperitoneally with sodium fluoroacetate (NaFAc) in saline at a dosage of 4 mg. per kg. of body weight. At various intervals of time groups of 6 mice were sacrificed and citrate determinations were made on their pooled lungs.

acid content had begun to decrease (Table II). From these data it was concluded that citric acid oxidation could be blocked in the lung of the mouse by the intraperitoneal injection of 4 mg. per kg. of sodium fluoroacetate. Further, the maximum period of blocking achieved by one injection at this dosage was something less than 12 hours. These results closely parallel those obtained by other workers who studied the action of fluoroacetate in rats (4, 9, 10).

Effect of Sodium Fluoroacetate on the Propagation of Influenza Virus in the Mouse Lung.—

Five groups, each containing 6 mice, were inoculated intranasally with 0.05 ml. of influenza virus, the infectivity titer of which for eggs was $10^{3.5}$. 15 minutes later they were injected intraperitoneally with 0.25 ml. of sodium fluoroacetate in saline. The concentration of fluoroacetate was varied so that graded dosages were administered to different groups of mice. 24 hours after the intranasal inoculation, the mice were sacrificed and their lungs were removed. The infectivity titer for eggs was determined on the pooled lungs from each group of mice. The titers of the lungs of the mice treated with fluoroacetate were compared with those of mice treated with saline.

Marked inhibition of virus propagation was observed with a dosage of 2 mg. per kg. of fluoroacetate. Maximum inhibition was achieved with 3 to 5 mg./K

TABLE III

Effect of Various Concentrations of Sodium Fluoroacetate on Propagation of Influenza Virus in Mouse Lung

Concentration of NaFAc	Virus concentration at 24 hrs. Egg infectivity titer*
mg./kg. of body weight	
0	6.23
2	5.00
3	4.00
4	3.83
5	4.30

All mice were injected intraperitoneally with 0.25 ml. of sodium fluoroacetate (NaFAc) in saline 15 minutes after intranasal inoculation with 0.05 ml. of influenza virus PR8 strain of titer $10^{3.5}$.

* Titer expressed as the log of the reciprocal of the dilution.

in which case the lowest infectivity titer of the lungs for eggs was $10^{3.8}$ while that of the control was $10^{6.2}$ (Table III). Thus, the intraperitoneal administration of sublethal concentrations of sodium fluoroacetate which are known to inhibit oxidation of citrate was found to inhibit the propagation of influenza virus in the mouse lung.

Effect of a Single Injection of Fluoroacetate on the Growth Curve of Influenza Virus.—

To determine the duration of the effect of a single injection of fluoroacetate on the propagation of influenza virus, the growth curve of the virus was determined in the lungs of mice which were treated with saline and compared with that obtained in mice treated with fluoroacetate. Ten groups, each containing 6 mice, were inoculated with 0.05 ml. of influenza virus which had an infectivity titer for eggs of $10^{3.5}$. 15 minutes later 5 groups of mice were injected intraperitoneally with saline and 5 groups were injected similarly with 4.3 mg. per kg. of sodium fluoroacetate. At each 12 hour interval thereafter, one group of mice treated with saline and one group treated with fluoroacetate were sacrificed. The titers of pooled samples of these mouse lungs were determined in eggs.

The greatest difference was at the end of 12 hours in which case the titers of virus from animals treated with saline differed from those treated with fluoroacetate by nearly 3 logs. Differences were observed at 12, 24, and 48 hours after injection of the inhibitor (Table IV). However, the effect was found to grow small with time until at 48 hours the difference in infectivity titers was only 0.7 log.

Effect of the Time of Administration of Sodium Fluoroacetate.

If the administration of fluoroacetate inhibits oxidation of citrate and interrupts the Krebs cycle which is supplying energy for viral synthesis, it should be possible to halt viral multiplication at any point of the growth curve by the injection of that inhibitor. To test for this experimentally, 5 groups containing 6 mice each were inoculated intranasally with 0.05 ml. of influenza virus with a titer of $10^{3.5}$ for eggs. Two groups were injected intraperitoneally

TABLE IV

Effect of Fluoroacetate on the Rate of Multiplication of Influenza Virus in the Mouse Lung

Time after viral inoculation	Infectivity titer of lungs*	
	Controls	Treated
hrs.		
0	1.3	2.0
12	3.3	0.5
24	5.0	3.0
36	—	5.0
48	7.0	6.3

All mice were injected intraperitoneally with 0.25 ml. of saline or sodium fluoroacetate in saline at a dosage of 4.3 mg. per kg. of body weight 15 minutes after intranasal inoculation with 0.05 ml. of influenza virus of titer $10^{3.5}$.

* Titer expressed as the reciprocal of the dilution found infective in eggs.

with saline a few minutes after the inoculation of virus. One of these groups was sacrificed after 12 hours and the other at 24 hours. The infectivity titer in eggs of virus from each group of mouse lungs was determined. The 3rd, 4th, and 5th groups of animals were injected with 4.3 mg. per kg. of sodium fluoroacetate at 0, 6, and 12 hours, respectively. 24 hours after the inoculation of virus, these animals were also sacrificed and the infectivity titers of virus in their lungs were determined.

From Table V it is seen that regardless of when the inhibitor is administered, there is inhibition of viral multiplication. Further, the titers of lungs of the control mice at 12 and 24 hours were $10^{4.0}$ and $10^{6.7}$, respectively, while the titer at 24 hours of the lungs of mice treated with inhibitor 12 hours after viral inoculation was $10^{3.7}$. This indicates that the inhibition produced by one injection of fluoroacetate was effective for approximately 12 hours.

Effect of the Concentration of the Viral Inoculum.—If the action of fluoroacetate is to render the chemical environment unsuitable for virus propagation,

then inhibition should occur to some extent irrespective of the amount of virus present. The data of Table V indicate this to be true since inhibition can be produced with fluoroacetate initially or after the virus has multiplied for 6 or 12 hours.

TABLE V

Effect of Time of Administration of Sodium Fluoroacetate on the Inhibition of the Propagation of Influenza Virus in Vivo

Time of intraperitoneal injection after inoculation of virus	Infectivity titer of lungs*		
	Controls		Treated
	12 hrs.	24 hrs.	24 hrs.
hrs.			
0	—	6.7	4.0
6	—	6.7	4.3
12	4.0	6.7	3.7

All mice were inoculated intranasally at zero time with 0.05 ml. of influenza virus of titer $10^{3.5}$. Sodium fluoroacetate (NaFAc) was administered intraperitoneally in 0.25 ml. of saline at a dosage of 4.3 mg. per kg. of body weight. Control animals were injected with a similar volume of saline.

* Titer expressed as the log of the reciprocal of the dilution found infectious for eggs.

TABLE VI

Effect of Sodium Fluoroacetate on the Propagation of Influenza Virus Induced by Various Concentrations of Inoculum

Virus titer* of inoculum	Infectivity titer of lungs at 24 hrs.	
	Controls	Treated
6.5	8.23	6.30
5.5	7.23	4.70
4.5	7.50	4.39
3.5	6.50	2.00

The intranasal inoculation of the mice with influenza virus of various titers was followed by intraperitoneal injection of sodium fluoroacetate (NaFAc) at a dosage of 4 mg. per kg. of body weight or with saline.

* Titer expressed as the log of the reciprocal of the dilution found infectious for eggs.

A further test was carried out by injecting mice with inocula of different concentrations and testing the mice for inhibition of virus multiplication by fluoroacetate. Four groups of 12 mice each, were injected intranasally with 0.05 ml. of influenza virus of titers $10^{6.5}$, $10^{5.5}$, $10^{4.5}$, and $10^{3.5}$, respectively. One-half of each group of mice was treated 15 minutes later with 4 mg. per kg. of sodium fluoroacetate. The second half of each group was treated with saline. At the end of 24 hours the 8 groups of mice were sacrificed and the virus content of their lungs was titered in eggs.

With each concentration of inoculum used, the titer of the lungs of the animals treated with fluoroacetate was 2 to 4 logs lower than the corresponding control group treated with saline (Table VI). It is evident that the degree of inhibition observed during a 24 hour period is greater as the concentration of virus inoculum is decreased.

DISCUSSION

When fluoroacetate is administered to animals, the enzyme system necessary for the oxidation of citrate is inactivated, while the enzymes necessary for synthesis of citrate appear to be relatively unaffected (5, 9–11). The continued synthesis of citrate without its further metabolism leads to the accumulation of this metabolite in large quantities in many tissues of the animal (4, 3). It has been suggested that the rise in citrate concentration is primarily an expression of the metabolism of the individual organ. This conclusion is supported by the fact that the citrate contents of the individual organs of the animals treated with sodium fluoroacetate differ widely from one another and from the blood level (2, 9, 10).

It has been reported that at relatively high concentrations, fluoroacetate will inhibit the oxidation of fatty acids which takes place in the presence of washed suspensions of rabbit liver probably owing to the binding of magnesium ions into a magnesium-fluoroacetate complex (12). The significance of this finding *in vivo* is as yet unknown. It is difficult to determine if the action of any inhibitor *in vivo* is specific. However, the problem of specificity does not alter the action of the inhibitor in the citric acid cycle.

By the administration of sublethal doses of fluoroacetate which were found to increase the concentration of citrate in the lungs of mice, it is possible to demonstrate a blocking of the citric acid cycle in those organs. Since these reactions are essential for viral synthesis as indicated by prior experiments *in vitro* (1), the propagation of influenza virus should be inhibited under these conditions in the lung. Experimentally this anticipated inhibition of viral propagation by fluoroacetate was verified (Tables II, IV, V, and VI).

The experiments described here are confined to one strain of influenza virus; however, it might be anticipated that fluoroacetate will act against a relatively broad spectrum of viruses. Firstly, the inhibitor blocks citrate metabolism not only in the lung but in the brain and spinal cord of mice. Secondly, it would seem that an energy source for endergonic synthetic reactions, *e.g.* the formation of nucleoprotein, is an essential requirement for the propagation of virus and may be one phase of viral synthesis which several host-virus systems have in common.

At present, data are not available to describe the effect of multiple injections of fluoroacetate on the course of a viral infection in mice. Indeed it is questionable whether the use of this inhibitor alone will be of value in chemotherapy.

The real worth of these experiments lies in pointing up one site in the host metabolism where some drug might act usefully. It is conceivable that an inhibitor can be found which will act at this site and in addition will possess other desirable properties which are essential for a useful drug. These data indicate that it is possible to achieve inhibition of viral synthesis in the intact mouse by the partial suppression of the Krebs cycle.

SUMMARY

A further study has been made of the relationship of reactions of the Krebs cycle to the propagation of influenza virus. By the administration of sublethal doses of sodium fluoroacetate which were found to increase the concentration of citrate in the mouse lung, it was possible to demonstrate a blocking of the citric acid cycle in that organ. Further, the intraperitoneal administration of these concentrations of fluoroacetate was found to inhibit markedly the propagation of influenza, Type A, virus in the lungs of mice.

The inhibition was observed when the fluoroacetate was administered 15 minutes, 6 hours, or 12 hours after the mice were inoculated with virus. This effect was also demonstrable when the concentration of the viral inoculum was varied over a range of virus titers from $10^{3.5}$ to $10^{6.5}$.

Sodium fluoroacetate was found to have no effect *in vitro* on the infectivity or hemagglutinating property of the virus.

The significance of these findings with regard to chemotherapy and to the mechanism of viral synthesis is described.

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