Action of Foot-and-Mouth Disease Virus and Metabolic Poisons on Bovine Kidney Culture Cells. (26191)

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Viral propagation is known to be dependent on host cell metabolism. Carbohydrate metabolism is important both as a source of energy and of carbohydrate intermediates which may serve as precursors in the viral synthetic process. Virus-induced increases in glycolysis have been observed with poliovirus (1), adenovirus(2), Newcastle disease virus (3), and foot-and-mouth disease virus (FMDV)(4), while infection with influenza virus has been reported to inhibit glycolysis of white blood cells(5). The importance of metabolic activities in the citric acid cycle has been demonstrated for influenza virus by deprivation of substrates, suppression of oxygen tension, interruption of the hydrogen transport system, and inhibition of a primary dehydrogenase(6). Development of poliovirus in mice has been inhibited by the presence of fluoroacetate which functions as a blocking agent of the citric acid cycle(7), but was not inhibited in HeLa cells by several amino acid and purine analogues(8). Anaerobic maintenance of host cells has inhibited the replication of mouse encephalomyelitis virus(9), and pneumonitis virus(10), while poliovirus was able to replicate(11).

The study reported here concerns the effect of various metabolic poisons on normal and infected bovine kidney culture cells. The poisons used are inhibitors of glycolysis, the citric acid cycle, and the cytochrome oxidative sequences. Infected cells are maintained aerobically and also anaerobically.

Materials and methods. Tissue culture. Primary monolayer cultures from bovine kidney tissue were prepared in 4-oz. prescription bottles as previously described(12). The growth medium consisted of 0.5% lactalbumin hydrolysate and 2% bovine serum in Hanks' balanced-salt solution. *Virus.* High-titer stocks of FMDV-A119 were produced from infected tissue culture fluids by precipitation with methanol, and the alcohol removed by subsequent dialysis(4). *Virus assay.* Virus assays were done by the plaque method using the monolayer cultures in prescription bottles(12). *Respiration.* Cell suspensions were prepared from cells scraped from the prescription bottles by packing them at 300 g for 5 minutes and resuspending in growth medium at concentrations of about 25×10^6 cells per ml. Warburg flasks contained 1 ml of cell suspension, 1 ml of isotonic Tris buffer, pH 7.5, containing the poison to be tested, and when needed, 0.50 ml of viral concentrate at 5-10 plaque forming units (PFU) per cell. Oxygen uptake was measured at 37°C with 0.20 ml of 10% KOH in the central well. *Virus production.* Cell layers in prescription bottles were rinsed twice with fresh portions of Tris buffer, then inoculated with 0.125 ml of viral concentrate (4-6 PFU per cell) and 0.125 ml of Tris buffer containing twice the desired final concentration of poison to be tested. At 90 minutes, the cells were washed 5 times with 5 ml portions of Tris buffer to remove most of the unadsorbed inoculum, and reincubated with 5 ml of growth medium-Tris buffer (1:1) containing the poison. At the desired time interval (100-150 minutes post inoculation for initial appearance of virus, and at 300 minutes for total virus production), 5 ml of 1% sodium dodecyl sulfate in Tris buffer was added to lyse the cells. The resulting lysate, which contained intracellular as well as extracellular FMDV progeny(4,13), was assayed for virus. In those experiments requiring an anaerobic environment, the prescription bottles were evacuated and flushed 5 times with nitrogen, then kept tightly stoppered. *Glucose-lactic acid.* Cell layers were inoculated as for virus production, but instead of the rinse at 90 minutes, 2.25 ml of growth medium-Tris buffer (1:1) containing the poison being tested were added and the bottles were rocked gently until 210 minutes post inocula-

TABLE I. Effect of Metabolic Poisons on Respiration of Suspensions of Kidney Culture Cells.*

Exposure time (hr)	% of control (no poison) respiration†							
	Iodoacetate		Fluoride		Malonate		Fluoroacetate	
	10^{-4}M	10^{-3}M	10^{-3}M	10^{-2}M	10^{-2}M	10^{-1}M	10^{-3}M	10^{-2}M
1	100	94	100	95	100	77	89	78
2	100	81	100	98	100	77	83	77
3	100	70	100	93	100	74	79	73
4	100	60	100	89	100	71	75	70
5	100	51	100	83	100	65	71	66

* Data are for both normal and infected cells.

† There was also no difference in respiration between normal and infected cells during first 5 hr after infection, in the absence of any poison. Normal respiration was 30 μl of oxygen/hr/25 $\times$ 10^6 cells(4).

tion. A 2.0 ml aliquot was then removed and treated with an equal volume of 10% tri-chloroacetic acid. The resulting supernatant liquids were assayed for glucose by the anthrone reagent(14) and for lactic acid(15).

Metabolic poisons. The chemicals were used as the sodium salts. Six replicates were done for chemical and viral asays, and 4 for respiration measurements.

Results. The effects on respiration of normal and infected cells in suspension exposed to varying concentrations of 4 different metabolic poisons were identical (Table I). 10^{-4}M iodoacetate, 10^{-3}M fluoride, and 10^{-2}M malonate had no effect on respiration over a 5-hour exposure period. Ten-fold higher concentrations of the same agents decreased respiration rates in all cases, the effect being progressive with 10^{-3}M iodoacetate and 10^{-2}M fluoride, while 10^{-1}M malonate caused a rapid initial drop in respiration followed by a slow continued decrease. Fluoroacetate also caused a relatively large initial drop with comparatively small differences in effect between the two concentrations used.

The effect of cyanide and azide was measured by extent of cell loosening from the glass culture vessel instead of attempting to maintain the poisons in the Warburg flasks in the recommended way(16). 10^{-2}M concentrations of these poisons did not influence cellular attachment to glass over the 5-hour period studied. At 5-fold higher concentrations, azide still had no effect by 5 hours, while cyanide caused partial cell loosening in 3 hours and complete cell loosening within 4 hours.

Table II gives the effect of 2 known poisons of the glycolytic cycle, iodoacetate and fluoride, on glucose utilization and lactic acid production in both normal and infected cells. Iodoacetate, at 10^{-3}M concentration, stopped glycolysis immediately in both classes of cells, while 10^{-4}M iodoacetate decreased glycolysis by half in less than 30 minutes, and thereafter to less than 5-6% of control values. Fluor-

TABLE II. Effect of Iodoacetate and Fluoride on Glucose Utilization and Lactic Acid Production of Bovine Kidney Cultures Cells, Normal and Infected. Percentage of control (no poison) values.*

Exposure time (min.)	Iodoacetate				Fluoride			
	10^{-4}M		10^{-3}M		10^{-3}M		10^{-2}M	
	Glucose	Lactic acid	Glucose	Lactic acid	Glucose	Lactic acid	Glucose	Lactic acid
Normal cells:								
30	—	53	—	0	—	100	—	85
210	10.5	9.1†	0	0	97	100	65	69
Infected cells:								
30	—	58	—	0	—	100	—	87
210	8.4	8.0†	0	0	95	100	42	46

* Control values, μg/hr/million cells, for normal and infected cells, respectively, were: glucose, 9.6 and 11.8; lactic acid, 5.3 and 10.2.

† There is still lactic acid production after first 30 min., equal to about 5 to 6% of control values.

TABLE III. Effect of Metabolic Poisons on Initial Appearance of Virus and Its Total Production in Bovine Kidney Culture Cells.

Poison	Virus yield, PFU/ml (log units)	Delay in initial virus appearance (min.)	Poison	Virus yield, PFU/ml (log units)	Delay in initial virus appearance (min.)
Normal control	7.3	—	Normal control	7.3	—
Iodoacetate,			Malonate,		
10^{-6}M	7.3	—	10^{-2}M	7.3	none
10^{-5}M	7.0	none	2.5×10^{-2}M	7.3	—
10^{-4}M	6.3	"	5.0×10^{-2}M	7.0	—
10^{-3}M	4.3*	—	1×10^{-1}M	6.7	10
Normal control	7.3	—	Normal control	7.8	—
Fluoride,			Fluoroacetate,		
10^{-3}M	7.3	none	10^{-4}M	7.8	—
5×10^{-3}M	6.4	—	1×10^{-3}M	7.8	0–10
1×10^{-2}M	5.8	none	5×10^{-3}M	7.3	—
5×10^{-2}M	4.4*	—	1×10^{-2}M	6.8	30
Normal control	7.3	—	Normal control	7.3	—
Cyanide,			Azide,		
10^{-3}M	7.1	0–10	10^{-3}M	7.2	0–5
5×10^{-3}M	6.6	—	5×10^{-3}M	7.1	—
1×10^{-2}M	6.0	30	1×10^{-2}M	6.4	40
5×10^{-2}M	4.2*	—	5×10^{-2}M	4.1*	—

* No virus production; titer represents residual viral inoculum(4).

ide, at 10^{-2}M concentration, decreased glycolysis to about two-thirds of control values in normal cells and had a greater effect in the virus infected cells, while 10^{-3}M fluoride had no appreciable effect on glycolysis in both normal and infected cells.

Table III gives the effect of the metabolic poisons on virus production in cells. Iodoacetate, at 10^{-3}M concentration, stopped all virus production; 10^{-3}M fluoride had no effect; 10^{-2}M fluoride lowered virus production by about 1.5 log units. Neither poison influenced time of initial appearance of virus. Fluoroacetate showed a stronger inhibitory effect than malonate on a molar basis, and both poisons delayed the onset of virus production. Cyanide showed a slightly more inhibitory effect than azide, and both poisons also extended the latent period of virus production.

Table IV shows the effect on virus production when the poisons were present only at selected time intervals during the virus growth cycle. The concentrations of poisons which decreased virus production when present throughout had a lesser effect when present only during the time period of 90-300 minutes. The effect of the presence of the poison solely during the first 90 minutes was negligible except in the case of iodoacetate. Absence of oxygen did not interfere with virus production.

Discussion. The poisons used fall into 3 categories based on the metabolic sequences they inhibit. Iodoacetate and fluoride affect enzymes of the glycolytic cycle, inhibiting triose phosphate dehydrogenase and enolase, respectively. Fluoroacetate and malonate affect enzyme substrates of the citric acid cycle, interfering with oxidation of citrate and of succinate, respectively. Cyanide and azide react with the heme containing enzymes of the cytochrome oxidation sequences. The virus itself was found to be stable in presence of all the poisons tested.

Decrease in respiration rate and extent of cell loosening can be used as a measure of the compatibility of the cells with the poisons present. The cells showed remarkable resistance to all the poisons used; respiration of the cells may be influenced more by other metabolic cycles than the citric acid cycle. Iodoacetate had the strongest effect, which may be explained by the fact that the poison is a non-specific inactivator of sulfhydryl groups, the integrity of which are necessary

TABLE IV. Effect on Virus Production of Poisons Present in Host Cells for Different Times during Virus Growth Cycle. Effect of anaerobic environment on virus production.

Poison		Virus titer, PFU/ml (log units)			
		Normal control	Present 0-300 min.	Present only 0-90 min.	Present only 90-300 min.
Iodoacetate,	1×10^{-4}M	7.7	6.6	7.0	6.9
	1×10^{-3}M	7.7	4.3*	5.4	5.5
Fluoride,	1×10^{-3}M	7.3	7.3	7.3	7.3
	1×10^{-2}M	7.7	6.1	7.6	6.5
Malonate,	1×10^{-2}M	7.3	7.3	7.3	7.3
	5×10^{-2}M	7.7	7.5	7.7	7.7
	1×10^{-1}M	7.3	6.6	7.3	7.1
Fluoroacetate,	5×10^{-3}M	7.6	7.1	7.4	7.3
	1×10^{-2}M	7.6	6.7	7.4	7.3
Cyanide,	1×10^{-3}M	7.4	7.3	7.4	7.4
	1×10^{-2}M	7.5	6.3	7.5	7.2
Azide,	1×10^{-3}M	7.5	7.3	7.5	7.5
	1×10^{-2}M	7.5	6.7	7.5	7.2
Nitrogen		7.5	7.5	7.5	7.5

* No virus production; titer represents residual viral inoculum(4).

for the action of numerous enzymes. The action of iodoacetate was also tested in presence of 10^{-2}M pyruvate. No change was noted. The decrease in respiration was therefore not caused by lack of pyruvate coming from the glycolytic scheme to be used in the citric acid cycle. Malonate had the least effect upon respiration with no effect at all at 10^{-2}M concentration. The impaired respiration with 10^{-1}M malonate may be attributed to the general effect of high salt concentration and not to a specific action. It would seem the bovine kidney culture cells may follow other oxidative sequences in addition to the citric acid cycle. Virus infection had no influence on respiratory response of the cells to the poisons, indicating that the presence of the virus did not weaken further the already damaged cellular systems.

Iodoacetate had a stronger effect than fluoride on glycolysis, and showed no difference in its action in the presence of virus. Fluoride, however, caused a greater decrease in glycolysis in virus-infected cells than in normal cells, which may indicate that virus infection had either changed or weakened a metabolic sequence affected by fluoride. Addition of pyruvate did not affect the action of either poison.

Virus production was affected more strongly by the glycolytic poisons, particularly by iodoacetate, than by any of the other groups of poisons. Only iodoacetate exercised a strong effect when present only during the 0-90 minute period after infection of the cells with virus. This may indicate that the effect of iodoacetate is irreversible, or that iodoacetate blocks all stages of viral replication—adsorption, penetration, synthesis and asembly of subunits, and discharge of intact virus. The action of the other poisons is apparently more reversible. It is of interest that the glycolytic poisons showed no effect on the latent period of virus production, while cyanide and azide produced a definite increase in latent period which may be interpreted as interference with the energy producing mechanisms required for virus synthesis. This interference may be associated with any heavy metal containing enzymes and not necessarily with the cytochrome oxidation chains. The greater effect on virus production shown by fluoroacetate as compared to malonate makes it probable that some other factor than interference with the oxidative steps of the citric acid cycle is involved. It has been reported that fluoroacetate causes a fall in concentration of free amino acids in treated tissues(17), and the effect of fluoroacetate upon viral propagation probably results from the diverting of viral precursors into synthesis of citrate(6).

The lack of effect of absence of oxygen during the 5-hour period of virus production is a further indication that oxidative sequences are not as vital to production of virus as are glycolytic sequences of the host cell.

Summary. Bovine kidney culture cells were found to be resistant to high concentrations of poisons of the glycolytic cycle, the citric acid cycle, and the cytochrome oxidation chain, as measured by respiration and cell loosening from the glass. Infection with FMDV had no influence on the results, and it may be that respiration of the cells is more dependent on other metabolic cycles. Iodoacetate and fluoride, present in concentrations which caused large decreases in glycolysis, decreased virus production more than any other poisons. Malonate had an insignificant effect on virus production, while the larger effect of fluoroacetate was probably due to its action in removing viral precursors. The fact that maintenance of infected cells in an anaerobic environment did not affect virus production was a further indication that glycolytic sequences of the host cell are more essential to viral replication than oxidative sequences.

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Studies of *Mycobacterium tuberculosis* $H_{37}Rv$ Grown *in vivo:* Inhibitor of Lactic Acid Dehydrogenase in Normal and Infected Mice. (26192)

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It has been previously reported(1,2) that intact cells and cell-free extracts of tubercle bacilli grown *in vivo* ($LH_{37}Rv$) inhibited lactic dehydrogenase in cell-free extracts of *Mycobacterium tuberculosis* $H_{37}Rv$, *Mycobacterium tuberculosis* BCG and *Mycobacterium phlei* as well as succinoxidase of lung homogenates of normal mice. Similar inhibition was displayed by lung extracts from mice infected with $H_{37}Rv$, but not by those from normal mice. The question arose whether the inhibitor was being produced by the tubercle bacillus itself or by the host tissues. Since the problem of the origin of the inhibitor is of great importance, it seemed essential to ascertain whether lung extracts from normal mice are completely devoid of inhibitory activity. Our findings that lung extracts from normal mice did not display any inhibitory activity do not preclude the possibility that the inhibitor in question was present in them but in quantity so small as not to be detected under our previous experimental conditions. This paper is concerned with the elucidation of this problem.

Materials and methods. The organism used was *Mycobacterium phlei.* The cells were grown at 37° on a rotary type shaker in a