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

Difco Nutrient Broth supplemented with Tween-80, 0.5%; sodium chloride, 0.5%; and K_2HPO_4, 0.25%. Three-day-old cultures were used. The cells were maintained on slopes with solid medium of a similar composition except for Tween-80. The cells of *M. phlei* were collected by centrifugation, washed twice in a 0.1M phosphate buffer, pH 7.2, and disintegrated in a 9 KC Raytheon magnetostriction oscillator for 45 minutes. Cell debris and broken cells were removed by centrifugation at 7700 × g for 10 minutes in the cold. The supernatant was used throughout. Protein content of the cell-free extracts was estimated by the biuret method. Mice were infected with *M. tuberculosis* $H_{37}Rv$ by the method described previously(2). For preparation of extracts from infected and normal mice, 2.5% homogenates were used. The various organs were homogenized in distilled water in a teflon homogenizer run at top speed for 2 minutes. The homogenates were then spun at 10,000 rpm in an MSE high speed centrifuge and the reddish opalescent supernatant was used. Dehydrogenase activity of cell-free extracts of *M. phlei* was determined by reduction of triphenyltetrazolium chloride under aerobic conditions. Formazan was extracted with *iso*-butanol and its color intensity read in a Coleman Jr. spectrophotometer at 485 mμ. Values are expressed in O.D. of formazan.

Results. Inhibition of lactic acid dehydrogenase of M. phlei *by lung extracts from normal mice.* To determine whether lung extracts from normal mice contain an inhibitor of lactic dehydrogenase of *M. phlei* we have performed a series of experiments using: 1) large amounts of lung extracts, 2) cell-free extracts of *M. phlei* of low dehydrogenase activity, and 3) preincubation of lung extracts with cell-free extracts of *M. phlei* before determining their lactic dehydrogenase activity.

1) Experiments with large amounts of lung extracts from normal and infected mice. In this series of experiments we determined the effect of increasing amounts of lung extracts from normal and infected mice on lactic dehydrogenase of cell-free extracts of *M. phlei.* The results (Table I) show that lung extracts from normal mice inhibit lactic dehydrogenase activity of cell-free extracts of *M. phlei* when used in amounts 2-3 times higher than those from infected animals. Six-tenths ml of lung extract from normal mice caused a 42% inhibition of lactic dehydrogenase of cell-free extracts of *M. phlei,* while a similar amount of lung extract from mice infected with $H_{37}Rv$ brought about an inhibition of 68%. Furthermore, it can be seen that the inhibitory activity of lung extract from infected mice is detectable at 2-3 fold higher dilutions than that of extracts from normal mice.

TABLE I. Effect of Various Amounts of Lung Extracts from Normal and Infected Mice on Lactic Dehydrogenase Activity of Cell-Free Extracts of *M. phlei.*

	Reduction of tetrazolium by cell-free extracts of *M. phlei* in presence of			
	Lung extracts from infected mice		Lung extracts from normal mice	
Amt of lung extracts used, ml	O.D. of formazan	Inhibition, %	O.D. of formazan	Inhibition, %
none	1.00		1.00	
.60	.32	68	.58	42
.40	.32	68	.74	28
.30	.42	58	1.00	0
.20	.66	34	1.10	stim*
.10	1.00	0	1.10	"

Reaction mixture contained: 6 mg protein of *M. phlei* extracts in 0.1M phosphate buffer pH 7.2, 0.5 ml; 0.3M sodium lactate, 0.1 ml; 1% aq. sol. of triphenyltetrazolium chloride, 0.1 ml; lung extracts as indicated. Final vol 2.2 ml. The reaction was stopped after 10 min. incubation at 37°.

* Stimulation.

2) Experiments with cell-free extracts of *M. phlei* of low dehydrogenase activity. It has been observed during these experiments, that lung extracts from normal mice inhibited lactic dehydrogenase in some batches of *M. phlei* preparations even when the former were used in small amounts (0.25 ml of lung extract per reaction mixture). The inhibition became apparent only with cell-free extracts of *M. phlei* of low dehydrogenase activity, (less than 0.7). Activity of lactic dehydrogenase is expressed in O.D. of formazan formed by cell-free extracts of *M. phlei* in presence of lactate in 10 minutes. In view of the above findings, experiments were designed to determine whether depending on the potency of *M. phlei* dehydrogenase, a

TABLE II. Effect of Lung Extracts from Normal and Infected Mice on Lactic Dehydrogenase Activity of Cell-Free Extracts of *M. phlei* of Various Enzymatic Activities.

Dehydrogenase activity of cell-free extracts of *M. phlei*		
Control	In presence of 0.25 ml of lung extract from normal mice	In presence of 0.25 ml of lung extract from infected mice
1.50	2.70	.90
1.00	1.15	.32
.50	.15	.07

Conditions as in Table I.

given amount of lung extract may either stimulate, inhibit, or have no effect on the action of the enzyme. The results showed this to be the case. In the experiment given in Table II we determined the effect of a given amount of lung extract from normal and infected mice on lactic dehydrogenase of cell-free extracts of *M. phlei* of various enzymatic activities. These were obtained by dilution of the enzyme. Activity of the undiluted enzyme was 1.5 and that of the 2 dilutions 1.0 and 0.5 respectively. A given amount of lung extract from normal mice stimulated the lactic dehydrogenase of high enzymatic activity/1.5/, had no effect when the enzymatic activity was 1.0, and exhibited a pronounced inhibitory effect on the same enzyme when activity of the latter was lowered to 0.5. On the other hand, lung extract from infected mice in amounts similar to those from normal animals inhibited even the most active preparations of lactic dehydrogenase of *M. phlei.*

3) Experiments on preincubation of cell-free extracts of *M. phlei* with lung extracts. The results described above show that there is an interplay between amount of inhibitor used and activity of lactic dehydrogenase. Presence of the inhibitor in lung extract from normal mice can be revealed either by using large amounts of the aforementioned extract or by using cell-free extracts of *M. phlei* of low dehydrogenase activity. The experiments to be described below show that it is possible to detect the inhibitor in lung extracts from normal mice using small amounts of lung extracts and cell-free extracts of *M. phlei* of high enzymatic activity. This can be achieved by preincubation of cell-free extracts of *M. phlei* with the lung extract before determining dehydrogenase activity of the former. In these series of experiments we incubated cell-free extracts of *M. phlei* (of high enzymatic activity) with small amounts of lung extracts from normal and infected mice (0.1 ml of extract) for various periods of time and then determined the effect of preincubation on lactic dehydrogenase activity of *M. phlei* by adding lactate and tetrazolium. The results of a typical experiment are shown in Fig. 1. At zero time, lung extract from normal mice stimulated dehydrogenase activity of *M. phlei* extracts by approximately 30%. Preincubation of lung extracts from normal mice with cell-free extracts of *M. phlei* resulted in a gradual decrease in lactic dehydrogenase activity of the latter. After 15 minutes of preincubation lactic dehydrogenase activity of *M. phlei* dropped to control level (cell-free extracts of *M. phlei* without lung extract). Further preincubation brought about an inhibition of lactic dehydrogenase which amounted to 63% after 1 hour. On the other hand, lung extract from infected mice inhibited lactic dehydrogenase of *M. phlei* by 31% at zero time. Preincubation for 15 and 25 minutes resulted in 86 and 90% of inhibition respectively.

Presence of inhibitor in various organs

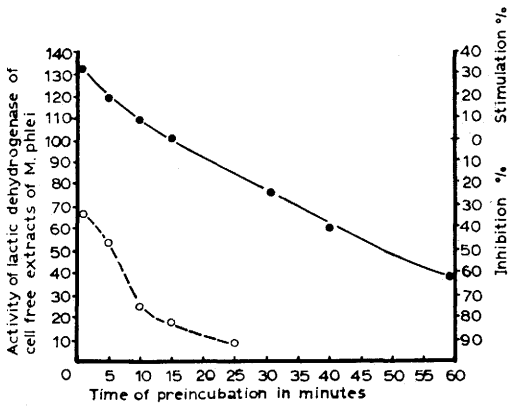

FIG. 1. Effect of preincubation with lung extracts from normal and infected mice on lactic acid dehydrogenase of cell-free extracts of *M. phlei.* Reaction mixture contained: 7 mg protein of bacterial extracts, 0.5 ml; lung extract, 0.1 ml; 0.1M phosphate buffer, pH 7.2, 0.5 ml; 0.3M sodium lactate, 0.1 ml; tetrazolium 1.0%, 0.1 ml. The reaction was stopped after 10 min. of incubation at 37°. Solid line (——), preincubation with lung extract from normal mice. Dotted line (– – –), preincubation with lung extract from infected mice.

TABLE III. Presence of Inhibitor of Lactic Dehydrogenase of *M. phlei* in Various Organs of Normal and Infected Mice.

Cell-free extracts of *M. phlei* in presence of	Reduction of tetrazolium by cell-free extracts of *M. phlei* O.D. of formazan			
	Without pre-incubation	With pre-incubation	Without pre-incubation	With pre-incubation
	Exp. I		Exp. II	
Control without lung extracts	.90	1.10	.98	1.20
Lung extract from infected mice	.32		.30	
Idem normal mice	1.20	.25	1.00	.12
Kidney extract from infected mice	.66		.90	.18
Idem normal mice	1.60	.40	1.75	.32
Liver extract from infected mice	.90	.32	.46	
Idem normal mice	1.60	.42	2.00	.28
Spleen extract from infected mice	.16		.08	
Idem normal mice	.50		.24	

Extracts of various organs were used in 0.25 ml amounts; for other conditions see Table I.

from normal and infected mice. In the light of the above findings it was of interest to examine various organs from both normal and infected mice for presence of the inhibitor. The results of 2 typical experiments (Table III) show that extracts from organs of normal and infected mice inhibit lactic dehydrogenase of *M. phlei* but to a different degree. Inhibitory activity of organs from infected mice was always higher than that of corresponding organs from normal animals. In the majority of cases inhibition of lactic dehydrogenase of *M. phlei* by extracts of organs from infected mice could be readily demonstrated (no preincubation was required). On the other hand, extracts of various organs from normal mice, with the remarkable exception of spleen, inhibited lactic dehydrogenase of *M. phlei* only after preincubation. Spleen extracts both from normal and infected mice inhibited lactic dehydrogenase of *M. phlei* without preincubation. It can be seen however, that the inhibitory activity of spleen extracts from infected mice was always much more pronounced than that from normal animals.

Discussion. It has been previously shown (1,2) that intact cells and cell-free extracts of the *in vivo* grown tubercle bacilli as well as lung extract from mice infected with $H_{37}Rv$ inhibited the electron transport system of various mycobacteria and of lung homogenates of normal mice. These findings posed the question as to the origin of the inhibitor. Experiments reported here show clearly that the inhibitor is present not only in the organs of infected mice but also in those of normal ones, which indicates that the origin of the inhibitor is the animal itself and that the inhibitory activity of tubercle bacilli grown *in vivo* is probably due to adsorption of the inhibitory substance on the surface of the bacillus. A marked difference in inhibitory activity of extracts from normal and infected mice was found. Extracts from mice infected with $H_{37}Rv$ always displayed a much more pronounced inhibitory activity than those from normal animals. It is clear therefore, that tuberculous infection is accompanied by, or causes an increase in inhibitory activity of the infected organs, probably by virtue of the stimulation of production of the inhibitor by tubercle bacilli. It is of great interest to elucidate the possible role of the inhibitor in the normal animal as well as in the tuberculous infected one (and possibly in other infections as well). Concerning the role of the inhibitor in the tuberculous process one may speculate that the inhibitor is introduced into phagocytes upon ingestion of tubercle bacilli coated with this substance. This process may bring the inhibitor in close contact with the respiratory enzymes of the phagocyte, thus causing inhibition of their respiration and eventually their death. Concerning the presence of the inhibitor in organs of normal mice, it would be of interest to determine whether it is in any way involved in regulation of respiratory

processes of the tissues. In connection with the role of spleen in the defense mechanism against infections, the presence of relatively large amounts of the inhibitor in this organ is noteworthy. These aspects are presently under investigation.

Summary. The presence of an inhibitor of the electron transport system of a number of mycobacteria and of lung homogenates of normal mice was found in normal mice and in those infected with *M. tuberculosis* $H_{37}Rv$. This inhibitor was previously found in tubercle bacilli grown *in vivo* and in lung extracts from infected mice. This work shows that the amount of inhibitor is greatly increased in animals infected with $H_{37}Rv$. The significance of these findings is discussed.

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Studies of *Mycobacterium tuberculosis* $H_{37}Rv$ Grown *in vivo:* Utilization of Glucose. (26193)

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Segal and Bloch(1) in their study on biochemical differences of *Mycobacterium tuberculosis* $H_{37}Rv$ grown *in vivo* and *in vitro,* compared the respiration of the 2 kinds of cells. They showed that both *in vitro* ($H_{37}Rv$) and *in vivo* ($LH_{37}Rv$) grown organisms had a similar rate of endogenous respiration but differed in their response to exogenous substrates. While respiratory response to glucose and its intermediates by $H_{37}Rv$ cells ranged from 59 to 102% depending on the substrate used, tubercle bacilli separated from lung tissues gave no response to any of these substrates above that of the endogenous. Discussing the failure of the $LH_{37}Rv$ cells to oxidize glucose and its intermediates, these authors suggested that this may be caused either by lack of permeability of $LH_{37}Rv$ cells to glucose or by an over-supply of endogenous food reserves. Although these factors would not readily explain the specific differential response to salycilic acid and fatty acids, neither of these possibilities was ruled out.

Reports from this laboratory(2,3,4) have shown that tubercle bacilli grown *in vivo* inhibit the electron transport system in cell-free preparations of various mycobacteria and in lung homogenates of normal mice. Inhibition of the electron transport system by tubercle bacilli grown *in vivo* is brought about by an as yet unidentified substance which has its origin in the host. This substance upon adsorption on the surface of the tubercle bacilli confers on them the inhibitory activity. In the light of these findings one can also assume that the inhibitor by coating the *invivo* grown tubercle bacilli interferes in some way with uptake and utilization of glucose by these cells. Since glucose is the only sugar present in animal tissues it seemed highly improbable that *in vivo* grown tubercle bacilli would be incapable of utilizing this important source of energy. We attempted, therefore, to answer this question by studying uptake and utilization of C^{14} Glucose by *in vivo* grown tubercle bacilli.

Materials and methods. The organisms used were: *Mycobacterium tuberculosis* $H_{37}Rv$ and *Mycobacterium tuberculosis* $LH_{37}Rv$. *Mycobacterium tuberculosis* $H_{37}Rv$ was grown and maintained in a liquid medium of the following composition: asparagine, 5.0 g; KH_2PO_4, 5.0 g; citric acid, 1.5 g; ferric ammonium citrate, 0.05 g; $MgSO_4$, 0.5 g; K_2SO_4, 0.05 g; glycerol, 0.5 g; Tween-80, 0.5 g; distilled water, 1,000 ml; bovine albumin to a final concentration of 0.25% was added after sterilization. The tubercle bacilli grown *in vivo* ($LH_{37}Rv$) were obtained from