STUDIES ON THE MULTIPLICATION AND THE PROPERTIES OF THE LACTIC DEHYDROGENASE AGENT

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While studying the relationship between tumor growth and plasma enzyme activity, Riley *et al.* (1) found a transmissible agent in the plasma of many tumor-bearing mice which upon injection into normal mice produced a five- to tenfold increase in the plasma activity of the enzyme lactic dehydrogenase (LDH). The increase in plasma LDH occurred within 72 hours after injection of this lactic dehydrogenase elevating agent (LDH agent) and persisted for months despite the lack of gross pathology. In addition to the enzyme LDH, Notkins *et al.* (2) found that isocitric dehydrogenase, malic dehydrogenase, phosphohexose isomerase, and glutamic-oxalacetic transaminase were also elevated. Further studies (2–4) revealed that much of the increased plasma enzyme activity which previously had been attributed to tumor growth was in fact due to the presence of this agent. Evidence from several laboratories suggested that the LDH agent was a virus (5–9) and was being carried as a contaminant (5, 9) by a number of transplanted mouse tumors. However, most of the work reported thus far has been concerned with the relationship of this agent to the tumor and plasma enzymes. Consequently there is very little detailed information in the literature on the nature and properties of the agent itself. The studies reported herein describe some of these properties and the procedure used to assay the LDH agent. The growth curve of this agent is described and findings pertaining to the incidence of cross-infection and transmission to offspring are presented.

Materials and Methods

LDH Agent.—The source and passage of the LDH agent has been described elsewhere (2). Two pools of infected plasma, designated P-9 and P-10, were obtained from mice 24 hours after they had been infected with the LDH agent. The infected plasma was put in individual vials, sealed under vacuum, and stored at −55°C until used.

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Diluents.—Eagle's minimum essential medium (10) with 20 per cent veal infusion broth (MEM-VIB)[1] was routinely used as the diluent for the LDH agent. In other experiments 0.1 M citrate-phosphate buffer (citric acid and dibasic sodium phosphate) at pH 4.0, 0.1 M phosphate buffer (monobasic potassium phosphate and dibasic sodium phosphate) at pH 7.3, 0.05 M carbonate-bicarbonate buffer (anhydrous sodium carbonate and sodium bicarbonate) at pH 10.3, and Dulbecco's phosphate-buffered saline (PBS) (11) with calcium and magnesium were used as diluents.

Plasma for Enzyme Assay.—Blood was obtained by orbital bleeding with heparinized micropipettes (12). Plasma was separated from the cells by centrifugation at 1500 *G* for 15 minutes and kept at 4°C until assayed. Hemolyzed specimens were not used.

Lactic Dehydrogenase Enzyme Assay.—The enzyme lactic dehydrogenase was assayed according to the method of Wroblewski and LaDue (13). One unit of enzyme activity is defined as the amount of enzyme which produces a decrease of 0.001 per 1 minute in the optical density of reduced nicotinamide-adenine dinucleotide ($NADH_2$) at 340 mμ.

Animals.—CAF-1 male mice, 4 to 6 weeks old and bred at the Animal Production Section of the National Institutes of Health, Bethesda, were used throughout the experiments.

Detection and Titration of the LDH Agent.—72 to 96 hours after injection of the LDH agent mice were bled and the units of enzyme (LDH) per milliliter of plasma were determined. Infected mice showed a five- to tenfold increase in plasma LDH while the plasma LDH from uninfected mice remained within the normal range. This five- to tenfold increase in enzyme activity served as the basis for detecting the LDH agent. Most enzyme determinations were done 72 to 96 hours after injection of the agent, but since the plasma LDH activity of infected mice was still elevated at the end of 1 year assays could be performed weeks and even months later. Specimens showing hemolysis were not used. Animals from which these specimens were obtained were simply bled again several days later. If doubt remained as to whether a particular animal was infected, the question was resolved by injecting its plasma into a normal mouse and then testing the latter for the characteristic increase in plasma LDH activity.

The infective titer of the LDH agent in a given preparation was determined by making serial tenfold dilutions. Except where indicated, Eagle's MEM-VIB was used as the diluent. Five mice served as recipients for each dilution and received 0.1 ml intraperitoneally. These animals were bled 72 to 96 hours later and tested for the five- to tenfold increase in plasma LDH activity. The number of animals infected at each dilution was determined and the ID_{50}/ml (dose that infected 50 per cent of the animals) was calculated by the method of Reed and Muench (14) and expressed on the basis of 1.0 ml of material.

RESULTS

Reproducibility of Assay Used for the Titration of the LDH Agent.—To determine the reproducibility of the assay, five completely separate sets of serial tenfold dilutions were made from a single lot of virus. PBS was used as the diluent in three of the assays and Eagle's MEM-VIB was used for the other two. As seen in Table I, the titer of the agent varied from $10^{8.7}$ to $10^{9.5}$ ID_{50}/ml with four out of the five values falling between $10^{8.7}$ and $10^{9.2}$ ID_{50}/ml.

Stability of LDH Agent.—The stability of the LDH agent was determined by storage in individual vacuum sealed vials at −55°C for 6 weeks. As seen in Table I, there was no appreciable loss of activity at the end of that time.

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[1] MEM-VIB, minimum essential medium with 20 per cent veal infusion broth.

TABLE I

Reproducibility of the Assay Used to Determine the Infective Titer of the LDH Agent

Material tested	Diluent	Assay No.	ID_{50}/ml (log 10)
Stock preparation of LDH agent (P-9)	PBS*	1	8.7
		2	8.7
		3	9.5
	Eagle's MEM-VIB‡	4	9.2
		5	9.0
Stock preparation of LDH agent (P-9) stored at −55°C for 6 wks.	PBS	1	8.6
	Eagle's MEM-VIB	2	8.7

* Dulbecco's phosphate-buffered saline.

‡ Eagle's minimum essential medium with 20 per cent veal infusion broth.

Effect of Dose of the LDH Agent on Plasma Enzyme Activity

To study the effect of different doses of the LDH agent on plasma enzyme activity, preparations containing $10^{1.5}$, $10^{4.5}$, and $10^{7.5}$ ID_{50}/ml were prepared in Eagle's MEM-VIB. Each mouse received 0.1 ml intraperitoneally of one of the above preparations and ten animals were used in each group. Control mice received 0.1 ml of diluent intraperitoneally. At 24, 48, 72, and 96 hours after injection the plasma LDH activity of each animal was determined. The individual LDH values were averaged and the standard deviation was calculated.

As indicated in Table II, all infected groups showed an increase in plasma enzyme activity over 96 hours. However, the mean plasma LDH level of those animals which received $10^{1.5}$ ID_{50}/ml was considerably lower at 48 hours than

TABLE II

Relationship of Dose of the LDH Agent to Plasma Enzyme Activity

Virus dose injected,* ID_{50}	Enzyme activity—units LDH/ml plasma‡			
	Hrs. after injection			
	24	48	72	96
Controls	290 ± 165§	466 ± 81	341 ± 134	480 ± 126
$10^{1.5}$	278 ± 164	1740 ± 363	3760 ± 328	4994 ± 408
$10^{4.5}$	325 ± 121	2590 ± 426	4215 ± 504	4905 ± 348
$10^{7.5}$	890 ± 106	3240 ± 494	4310 ± 475	5375 ± 569

* Injected intraperitoneally.

‡ Each figure represents the average of 10 mice.

§ ± standard deviation.

the mean of those which had received $10^{7.5}$ ID_{50}/ml. At 96 hours the plasma LDH activity of the three groups was approximately the same. Thus, the higher the infective dose, the more rapid the initial increase in plasma enzyme activity.

Growth Curve

Donor mice were injected intraperitoneally with $10^{5.9}$ ID_{50} of the LDH agent. At intervals thereafter, the donors were bled in groups of five. Exactly 50 μl of plasma from each of the five donors was pooled and 0.2 ml of this pool was added to 1.8 ml of Eagle's MEM-VIB to make a 10^{-1} dilution. Serial tenfold dilutions were continued in Eagle's MEM-VIB and 0.1

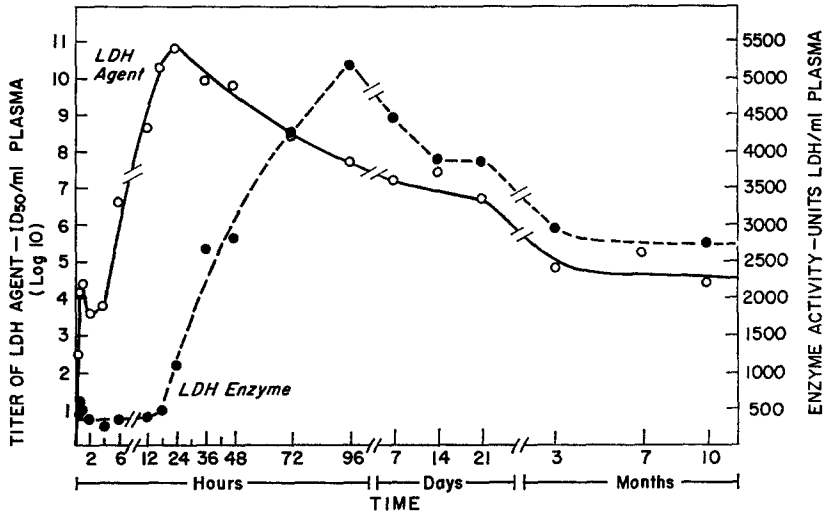

FIG. 1. Growth curve of the LDH agent and changes in the plasma activity of the enzyme LDH.

ml of each appropriate dilution was injected intraperitoneally into recipient mice. 72 to 96 hours later these recipients were bled and the plasma was assayed for enzyme activity (units LDH/milliliter). The number of animals infected was determined and the ID_{50} of the donor plasma was calculated.

As seen in Fig. 1, the injected virus was recovered from the plasma of donor mice within 5 minutes after intraperitoneal injection. At 6 hours the infective titer in the plasma was $10^{6.6}$ ID_{50}/ml, indicating that multiplication of the LDH agent had taken place (since the titer per milliliter of donor plasma was higher than the initial inoculum). The titer of the LDH agent then steadily increased, reaching $10^{10.8}$ ID_{50}/ml within 24 hours. Over the next 72 hours the plasma titer fell 3 logarithms to $10^{7.8}$ but decreased only 1 additional logarithm to

$10^{6.7}$ by the end of the 3rd week. At 10, 12, and 16 months the titers were $10^{4.4}$, $10^{4.3}$, and $10^{5.2}$ ID_{50}/ml respectively.

The enzyme activity of donor plasma was determined on the same samples used to titrate the LDH agent. Each point on the enzyme curve in Fig. 1 represents the average of individual determinations from five mice. The increase in plasma enzyme activity lagged 18 to 24 hours behind virus multiplication. At 24 hours when the virus titer was at its peak, plasma enzyme activity was only slightly above normal. At 96 hours when the plasma enzyme activity had reached its highest point, the titer of the LDH agent had fallen by 3 logarithms. At 10 months when the infective titer of the LDH agent had decreased by 6 logarithms, the plasma LDH activity had decreased by only 50 per cent.

Properties of the LDH Agent

Freezing and thawing.

A stock preparation of the LDH agent was diluted 10^{-2} in PBS, frozen in dry ice and acetone, and then thawed at 37°C. This procedure was carried out five times within a 20 minute period. Serial tenfold dilutions were made in PBS and the infective titer was determined.

As seen in Table III, freezing and thawing five times produced a decrease in the infective titer in only one out of the three experiments.

TABLE III

Effect of Freezing and Thawing, Lyophilization, and Ether on the Infective Titer of the LDH Agent

Treatment	ID_{50}/ml (log 10)
Stock preparation of LDH agent	8.9
Freezing and thawing*	8.6
	7.5
	8.5
Lyophilization	
Stored at 4°C for 4 days	8.6
Stored at 4°C for 90 days	8.5
Stored at 22°C for 17 days	9.2
Stored at 22°C for 90 days	7.7
Ether	
15 per cent at 4°C for 24 hrs	0
15 per cent at 4°C for 15 min	0

* Values from three separate experiments.

Lyophilization.

A stock preparation of the LDH agent was diluted 10^{-2} in Eagle's MEM-VIB. 1 ml was placed in individual vials, frozen in dry ice and acetone, and lyophilized overnight. The vials

were sealed under vacuum and stored in the dark at either 4°C or 22°C. At various times thereafter, vials were opened and reconstituted to the original volume with distilled water. Serial tenfold dilutions were made in Eagle's MEM-VIB and the infective titers were determined.

As seen in Table III, lyophilization and storage at 4°C for as long as 90 days did not alter the infective titer. However, storage at 22°C for 90 days did result in some loss of activity.

Treatment with ether.

To a preparation of the LDH agent containing $10^{6.9}$ ID_{50}/ml in Eagle's MEM-VIB, 15 per cent anesthetic ether (Mallinckrodt Chemical Works, St. Louis) was added. The test tube was stoppered, shaken, and kept at 4°C for 24 hours. Nitrogen was then bubbled through the tube to remove the ether. A control tube, unexposed to ether, was handled similarly.

As seen in Table III, intraperitoneal injection of 0.1 ml of the ether-treated material into recipient mice failed to produce infection. Further experiments revealed that treatment with 15 per cent ether for as little as 15 minutes at 4°C resulted in complete loss of infectivity if that material was shaken vigorously several times during the incubation period. If however, the ether was simply layered over the surface of the aqueous material without shaking, infectivity was not lost at the end of 15 minutes.

Effect of temperature.

A preparation of the LDH agent was diluted 10^{-2} in PBS and was incubated at 4°C, 22°C, and 37°C for 24 hours; 60°C and 80°C for 30 minutes; and 100°C for 2 minutes. After incubation, serial tenfold dilutions were made in PBS and the infective titer of each preparation was determined.

TABLE IV

Effect of Temperature on the Infective Titer of the LDH Agent

Temperature	Incubation time	ID_{50}/ml (log 10)	
		Experiment 1*	Experiment 2‡
Stock§	0	8.9	10.5
4°C	24 hrs.	9.0	9.8
22°C	24 hrs.	9.0	9.5
37°C	24 hrs.	7.7	7.8
60°C	30 min.	—	3.5
80°C	30 min.	0	0
100°C	2 min.	0	0

* Stock preparation P-9 was used.

‡ Stock preparation P-10 was used.

§ The stock preparations were diluted 10^{-2} in phosphate-buffered saline prior to incubation.

As seen in Table IV the LDH agent was stable at 4°C for 24 hours but was partially inactivated at 37°C for 24 hours. Almost total loss of activity occurred at 60°C for 30 minutes and complete inactivation took place at 80°C for 30 minutes and 100°C for 2 minutes.

Effect of diluents.

To compare the effect of different diluents on the infective titer of the LDH agent, a stock preparation was diluted 10^{-2} in Eagle's MEM-VIB, PBS, distilled water, phosphate, citrate-phosphate, and carbonate-bicarbonate buffers. These preparations were allowed to stand at 4°C for 3 hours and then serial tenfold dilutions were made with the original diluent.

TABLE V

Infective Titer of the LDH Agent After Incubation at 4°C for 3 Hours in Different Diluents

Diluent*	pH‡	ID_{50}/ml ($\log_{10}$)	
		Experiment 1	Experiment 2
Eagle's MEM-VIB§	7.3	10.5	10.4
Distilled water‖	6.6	—	10.4
PBS¶	7.3	—	9.7
Carbonate-bicarbonate buffer	10.3	9.3	9.4
Phosphate buffer	7.3	8.8	8.5
Citrate-phosphate buffer	4.0	7.6	7.6

* A stock preparation of the LDH agent was diluted 10^{-2} in each diluent and incubated at 4°C for 3 hours. The infective titer was determined by using the same diluent to do serial tenfold dilutions (except distilled water).

‡ pH measurements were made on the diluents after the LDH agent had been added.

§ Eagle's minimum essential medium with 20 per cent veal infusion broth.

‖ After incubation in distilled water, the serial tenfold dilutions were made in Eagle's MEM-VIB.

¶ Dulbecco's phosphate-buffered saline.

As seen in Table V, the highest titer was obtained when the LDH agent was diluted in Eagle's MEM-VIB. Substantially lower titers were found with phosphate and citrate-phosphate buffers.

Sedimentation of the LDH agent.

Modification of the procedure described by Moloney to concentrate virus from plasma was employed to sediment the LDH agent (15). 24 hours after intraperitoneal injection of the LDH agent, mice were anesthetized and heparinized blood was obtained from the axilla. Plasma was separated from the cells by centrifugation at 1500 *G* for 20 minutes, diluted with 0.3 M potassium citrate containing 3.0 mg per cent hyaluronidase, and was centrifuged at 2500 *G* for 15 minutes and at 10,000 *G* for 2 minutes. The infective titer of the LDH agent in the plasma was then determined and the final centrifugation was performed at

105,000 *G* for 2 hours in a Spinco model L Ultracentrifuge with a 40 rotor. 1 ml of the resulting supernatant fluid was withdrawn by inserting a 26 gauge needle through the side of the cellulose centrifuge tube at a point one-third of the distance from the top and the infective titer was determined. The remainder of the supernatant fluid was poured off and the tube drained. A pellet about 1.0 mm in diameter was seen and resuspended to the original volume. The infective titer of the resuspended pellet was determined.

As seen in Table VI, most of the virus was recovered in the resuspended pellet. Less than 0.1 per cent of the LDH agent remained in the supernatant fluid.

TABLE VI

Sedimentation of the LDH Agent

Materials assayed for infectivity*	ID_{50}/ml ($\log_{10}$)
Before ultracentrifugation	
Infected plasma	10.5
After ultracentrifugation	
Resuspended pellet	9.8
Supernatant fluid	6.6

* The infected plasma containing $10^{10.5}$ ID_{50}/ml was centrifuged at 105,000 *G* for 2 hours An aliquot of the supernatant fluid was removed and assayed for infectivity. The virus pellet was then resuspended to the original volume and assayed for infectivity.

Estimation of size of the LDH agent.

In cooperation with Dr. Wallace Rowe of the National Institute of Allergy and Infectious Diseases, gradocol membrane studies were performed. Infected plasma titering about $10^{6.0}$ ID_{50}/ml was diluted 10^{-2} in Eagle's MEM-VIB, centrifuged at 1500 *G* for 20 minutes, and then passed through an 800 mμ Millipore filter. Aliquots of the Millipore filtrate were passed through gradocol membranes of 320, 140, 110, and 74 mμ pore size. The gradocol membrane filtrates were tested for infectivity. The materials which had passed through the 320, 140, and 110 mμ membranes all infected mice, but that which was filtered through the 74 mμ membrane failed to infect mice.

Because of factors such as clogging of the pores and adsorption to the membrane, determination of particle size by this procedure is at best a rough approximation. According to Elford (16) the size range of a virus is one-third to one-half of the smallest pore size through which it passes. On this basis the maximum size of the LDH agent would be 55 mμ. However, if the correction factor suggested by Markham (17) is used, the size of the particle would be somewhat larger. A separate isolate of the LDH agent, obtained by Dr. Rowe in his laboratory, passed through a 110 mμ membrane, partially passed through an 85 mμ membrane, but failed to pass a 75 mμ membrane (18). Electron microscopic studies of the LDH agent are in progress.

Transmission of the LDH Agent

Transmission to cagemates.

Because of the association of the LDH agent with over 40 different types of mouse tumors (1, 5, 7–9) it was of interest to see if this agent would spread from infected to uninfected mice kept in the same cage. Five mice were placed in each cage and injected with the LDH agent. An equal number of normal (uninjected) mice were immediately placed in the same cage. All animals were numbered and at appropriate intervals thereafter, both injected and uninjected mice were bled and the number of newly infected animals was determined.

In the first experiment (Table VII) none of the normal mice had become infected within the first 45 days and only 2 were infected at 120 days. (The late infection of these two mice raises the possibility that accidental contamination of the blood collecting pipettes during one of the earlier bleedings might

TABLE VII

Transmission of the LDH Agent to Cagemates

After injection	Experiment 1*	Experiment 2
days		
3	0/20	—
17	0/20	—
30	—	1/20
45	0/20	—
75	—	1/20
120	2/20	—

* On day zero, 20 mice were infected with the LDH agent. An equal number of normal (uninfected) mice were placed in the same cages. At intervals thereafter the normal cagemates were examined for infection. The figures represent the number of newly infected mice/original number of uninfected mice.

have resulted in orbital inoculation of the LDH agent and infection). In the second experiment only one out of twenty normal mice became infected.

Since less than 10 per cent of the animals had become infected, it was important to determine whether the remaining uninfected animals were still susceptible to the LDH agent or whether they had developed resistance to the agent as a result of prolonged exposure to the infected mice. A plasma pool was obtained from the infected mice, diluted 10^{-1} in Eagle's MEM-VIB and injected intraperitoneally into the uninfected mice of the same cage. All of these animals became infected within 72 hours. Although the incidence of cross-infection by ordinary contact was relatively low, the mice were readily susceptible to the LDH agent by the parenteral route.

Transmission to offspring.

Five adult male and five adult female mice were injected intraperitoneally with a preparation of the LDH agent containing $10^{5.9}$ ID_{50}. 1 week later these animals were mated and the females were placed in separate cages. Approximately 1 month after birth the number of offspring infected was determined.

As seen in Table VIII, only one out of the forty offspring became infected. However, experiments in progress indicate that inoculation of mice with the LDH agent during pregnancy (rather than prior to pregnancy) leads to an extremely high per cent of infected offspring. These studies will be reported separately.

TABLE VIII

*Transmission of the LDH Agent to Offspring from Mothers Infected Prior to Pregnancy**

Litter No.	No. mice infected/total mice per litter
1	0/7
2	0/7
3	1/7
4	0/9
5	0/10

* Both parents were infected with the LDH agent prior to mating. Offspring were examined for infection approximately 1 month after birth.

DISCUSSION

In studying the relationship between virus multiplication and plasma enzyme activity it was noted that at the height of viremia ($10^{10.8}$ ID_{50}/ml) the plasma LDH activity had increased by less than twofold. At 96 hours when the plasma LDH activity was at its height (ten- to twelvefold above normal), the virus titer had already decreased by more than one thousandfold. Thus, the appearance of virus in the circulation preceded the increase in plasma LDH activity. However, because of such factors as the rate of release of an enzyme from a cell and the kinetics of clearance of enzymes from the plasma, it is difficult to evaluate critically the significance of this time-lag or to determine the exact relationship between virus multiplication and the increase in plasma LDH activity. The mechanism by which the LDH agent produces the increase in plasma enzyme activity still remains obscure, but it has been suggested (2, 4) that the LDH agent may act by stimulating cellular enzyme production or producing cell damage and subsequent release of enzyme into the circulation. Although it is not unreasonable to postulate that the enzyme may be released from the same cell as that in which the virus grows, another possibility is suggested by the above data. Virus or viral by-products may enter the circula-

tion and act upon or damage a second cell (target cell) from which the enzyme is actually released. If the number of these target cells were few as compared to the number of virus particles, a low titer of virus might completely saturate these cells and produce a maximal rise in plasma enzyme activity. The excess virus particles over the saturation number would not lead to any further increase in plasma LDH activity. This explanation could account for the less than 50 per cent decrease in plasma enzyme activity at 10 months, although the titer of the LDH agent had dropped by over one millionfold. This could also explain the relatively constant plasma enzyme elevation (five- to twelvefold above normal) following infection as compared to the marked changes in virus titer.

The plasma titer of the LDH agent reached $10^{10.8}$ ID_{50}/ml within 24 hours after infection, but fell to $10^{4.8}$ ID_{50}/ml by the end of 3 months. The plasma titer remained at approximately that level for the next 13 months. A persistent viremia of this duration is unusual and suggests that the host does not have a completely adequate defense against the LDH agent.

Recent studies in our laboratory showed that the LDH agent was present in the saliva, urine, and feces of infected mice (19). The titer as compared to plasma was relatively low and virus was recovered only early in the course of infection. In addition, it was possible to infect mice by the oral route, but only if a high concentration of the LDH agent was used. The spread of the LDH agent from infected to uninfected mice housed in the same cages was low (Table VII). This low incidence of infection by ordinary contact and the failure thus far to find the LDH agent in normal mice suggests that the serial parenteral administration of contaminated biological materials, such as plasma, organs, or tumors from infected animals, may be the major factor responsible for the persistence of this agent in the laboratory setting.

The high plasma titer, the persistent viremia, the rapid and prolonged elevation of a number of plasma enzymes, and the lack of gross pathology add to the list of unusual properties which are displayed by the LDH agent in mice.

SUMMARY

The procedure used to determine the infective titer of the LDH agent, the reproducibility of this assay, and the relationship between virus dose and plasma enzyme activity were described. Multiplication of the LDH agent began within 6 hours after infection and reached $10^{10.8}$ ID_{50}/ml of plasma within 24 hours. The titer rapidly decreased over the next 72 hours but viremia persisted for at least 16 months with titers as high as $10^{5.2}$ ID_{50}/ml. The appearance of the LDH agent in the circulation preceded the first noticeable rise in plasma LDH activity by close to 24 hours. After 10 months, when the plasma titer of the LDH agent had decreased nearly one millionfold, the plasma enzyme LDH had decreased by less than 50 per cent.

The LDH agent is inactivated by ether but withstands lyophilization, and freezing and thawing. It is stable at low temperatures. Ultracentrifugation at 105,000 *G* for 2 hours leaves less than 0.1 per cent of the LDH agent in the supernatant fluid and filtration through gradocol membranes suggesst that the upper size of the LDH agent is about 55 mμ.

Spread of the LDH agent from infected to uninfected mice kept in the same cage and transmission from mothers (infected prior to mating) to their offspring was relatively low.

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