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Lactic Dehydrogenase Virus

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INTRODUCTION

In search of a simple diagnostic procedure for the early detection of cancer, many investigators have looked for differences in the enzyme activity of the blood of normal and tumor-bearing animals. In 1954, Hill and Levi (25) found that the enzyme lactic dehydrogenase (LDH) was elevated in the blood of a number of patients with malignancies. Shortly thereafter, similar findings were reported in tumor-bearing mice (34, 54). In 1960, while studying the relationship between enzyme elevation and tumor growth, Riley (55) found that, when he inoculated plasma or cell-free extracts from the tissues of tumor-bearing mice into normal mice, the recipients developed a 5- to 10-fold increase in plasma LDH activity within 48 hr. The enzyme elevation persisted for months, despite the fact that the animals failed to develop tumors or show any signs of illness or gross pathology. Riley showed that this agent which elevated plasma LDH replicated in mice and could be serially transmitted. Most important was the fact that, of the 26 different types of mouse tumors originally studied, all contained this lactic dehydrogenase agent (55). The widespread distribution of this agent initially suggested that it might be integrally related to the neoplastic process (55). However, reports from a number of laboratories soon revealed that it was not associated with all serially transplanted tumors (1, 36, 37, 47, 71), was rarely recovered from primary mouse tumors, and was never found in rat tumors (37). Further studies showed that the Moloney virus (which induces leukemia in both mice and rats) produced a 5- to 10-fold increase in plasma LDH activity in mice but not in rats (37). When the Moloney leukemia virus was passed in rats and injected back into mice, it produced the usual leukemia in mice but was no longer able to produce the enzyme elevation. Thus, it became apparent that the Moloney leukemia virus had been contaminated with the lactic dehydrogenase agent and that the latter agent had been eliminated by rat passage because of species specificity (37). These and subsequent studies showed that the lactic dehydrogenase agent was a virus (9, 40, 42), and was not integrally related to the Moloney leukemia virus or to the neoplastic process but was simply being carried as a contaminant by a number of serially transplanted tumors. The present review summarizes what is known about the lactic dehydrogenase virus (LDV), discusses the factors that might be responsible for its unusual manifestations, and considers the role that this widely disseminated, but previously unsuspected, virus might have played in the results and interpretations of a number of experiments.

ASSAY

The 5- to 10-fold increase in the activity of the enzyme LDH in the plasma of mice after inoculation of LDV served as the basis for detecting the virus. Most assays were performed within 96 hr after injection of the virus, but, since the enzyme elevation persisted for the life of the animal, assays could be performed weeks and even months later (40). The titer of LDV was determined by injecting serial 10-fold dilutions into recipient animals (40). The number of animals that became infected at each dilution was recorded, and the dose that infected 50% of the animals (ID_{50} per milliliter) was calculated by the method of Reed and Muench (53).

Studies on the relationship between virus dose and the increase in plasma enzyme activity (40)

TABLE 1. *Relationship of dose of LDV to plasma enzyme activity**

Virus dose injected (ID_{50})	Time after injection			
	24 hr	48 hr	72 hr	96 hr
Controls	290 ± 165	466 ± 81	341 ± 134	480 ± 126
$10^{1.5}$	278 ± 164	1,740 ± 363	3,760 ± 328	4,994 ± 408
$10^{4.5}$	325 ± 121	2,590 ± 426	4,215 ± 504	4,905 ± 348
$10^{7.5}$	890 ± 106	3,240 ± 494	4,310 ± 475	5,375 ± 569

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revealed that the greater the inoculum the more rapid was the increase in LDH activity (Table 1). However, after 96 hr, the plasma LDH level of animals that had received a low dose of virus ($10^{1.5}$ ID_{50}) was equal to that of the animals that had received a high dose ($10^{7.5}$ ID_{50}). Thus, regardless of the initial dose, once an animal became infected with LDV that animal developed the characteristic 5- to 10-fold increase in plasma LDH activity.

PHYSICAL AND CHEMICAL TREATMENT

The effect of physical and chemical treatment on the infective titer of LDV (40) is summarized in Tables 2 to 4. LDV was relatively stable to storage, lyophilization, and freezing and thawing (40). However, the virus was completely inactivated by ether, chloroform, and butanol (40, 46*a*). In addition, Georgii found that the virus was inactivated by formalin, alcohol, and *p*-aminosalicylic acid (20). Incubation of LDV at 37 C for 24 hr resulted in the loss of more than 99% of the infectivity, and complete inactivation occurred within 30 min at 80 C (40). Incubation of the virus at 4 C for 3 hr with 0.1 M sodium phosphate buffer (*p*H 7.3) or 0.1 M citrate-phosphate buffer (*p*H 4.0) decreased the infective titer by 2 to 3 logarithms. The highest titers were obtained in Eagle's medium with 20% veal infusion broth (40). Thus, the infective titer varies considerably with the particular diluent used, and this might account for the somewhat lower titers obtained in other laboratories.

SIZE ESTIMATION AND ELECTRON MICROSCOPY

Filtration through gradocol membranes indicated that LDV was between 30 and 55 mμ in diameter (40, 65). In a sucrose gradient, LDV had a density of 1.168 (61), a sedimentation coefficient of about 150, and a calculated spherical diameter of approximately 40 mμ (Riley, *personal communication*). Electron microscopic examination of a thin section of an osmium-fixed pellet prepared from viremic plasma (Fig. 1 and 2) revealed rounded particles averaging 40 mμ in

TABLE 2. *Effect of physical and chemical treatment on the titer of LDV (40)*

Treatment	ID_{50}/ml (log 10)
Stock	8.9
Stored at −55 C for 6 weeks	8.6
Lyophilized and stored at 4 C for 90 days	8.5
Lyophilized and stored at 22 C for 90 days	7.7
Freezing and thawing five times	8.6
Ether*	ND†
Chloroform*	ND
Butanol*	ND

* Shaken with an equal volume of solvent at room temperature for 3 min four times.
† None detected.

TABLE 3. *Effect of temperature on the titer of LDV (40)*

Temp	Incubation time	ID_{50}/ml (log 10)
C		
Stock	0	10.5
4	24 hr	9.8
22	24 hr	9.5
37	24 hr	7.8
60	30 min	3.5
80	30 min	ND*
100	2 min	ND

* None detected.

TABLE 4. *Infective titer of LDV after incubation at 4 C for 3 hr in different diluents (40)*

Diluent	*p*H	ID_{50}/ml (log 10)
Eagle's with 20% veal infusion broth	7.3	10.4
Dulbecco's phosphate-buffered saline with Ca and Mg	7.3	9.7
Carbonate-bicarbonate buffer (0.05 M)	10.3	9.4
Sodium phosphate buffer (0.1 M)	7.3	8.5
Citrate-phosphate buffer (0.1 M)	4.0	7.6

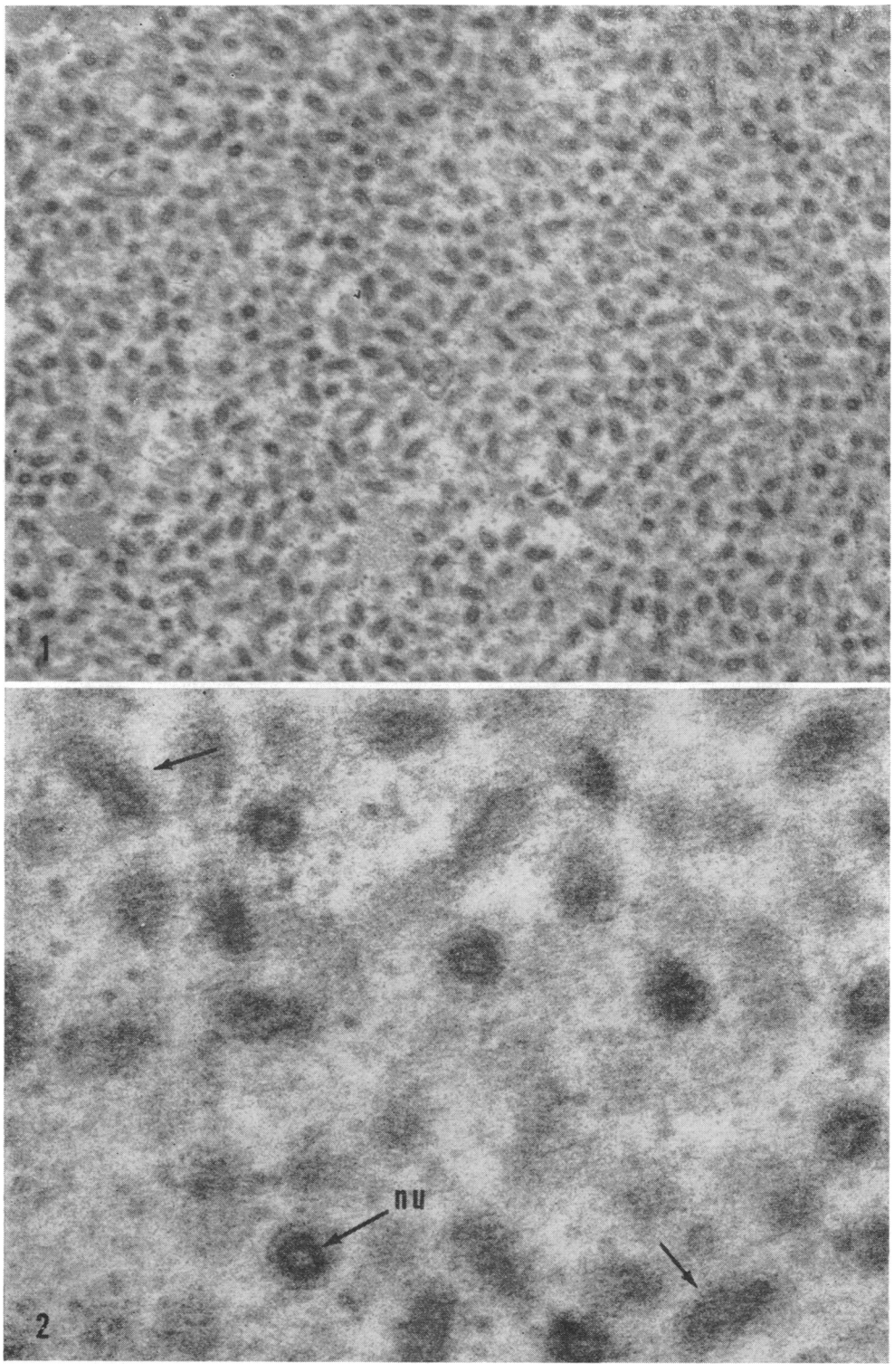

FIG. 1. *Thin section of a pellet prepared from the plasma of mice 24 hr after injection of LDV. A high concentration of particles can be seen (fixed with osmic acid).* × *60,000. Courtesy of Guy de-Thé.*

FIG. 2. *Same material as in Fig. 1, except higher magnification. The rounded particles are about 40 mμ in diameter and have a prominent nucleoid (nu) with a central core. A number of elongated forms (arrows), 45 to 75 mμ in length, can also be seen.* × *255,000. Courtesy of Guy de-Thé.*

diameter and elliptical or oblong particles 36 to 42 mμ wide and 45 to 75 mμ long (17*a*). The particles have a prominent ring-shaped nucleoid, 26 to 29 mμ in diameter, which consists of a dense ring and an electron-lucent core. Around the nucleoid is a 5 to 7 mμ thick layer limited by what appears to be a thin outer membrane. Particles similar in size and shape were found within macrophages obtained from the peritoneal cavity of infected mice (17*a*). If, however, pellets prepared from viremic plasma were not fixed with osmium tetroxide but instead were suspended in distilled water and negatively stained with phosphotungstic acid, the particles appeared larger and more pleomorphic with tail-like projections (9). Further studies showed that fixation with osmium tetroxide prior to staining substantially reduced the number of these forms, suggesting that they were artifacts produced in the preparation and staining of the virus (9). Thus, the morphological appearance of LDV is affected by the method of preparation. Similar variations in size and shape have been reported with other viruses (11, 17, 72).

In addition to the particle described above, several investigators (2, 16, 58, 60, 61) have suggested that a fraction of the particles (0.1% or less) might be considerably smaller or less dense. On the basis of diffusion measurements in a multilayered cell and of filtration studies, Riley (60) suggested that LDV might be as small as 2 mμ. Other evidence for a small particle comes from column chromatography experiments which showed that 0.1% of the original material did not elute with the main virus peak. Similarly, prolonged ultracentrifugation (105,000 × *g* for 20 hr) failed to remove all of the virus from the supernatant fluid (2, 61). Although the supernatant fluid contained between $10^{4.0}$ and $10^{7.0}$ ID_{50} per milliliter, this represented less than 0.1% of the starting material. Riley et al. (61) and Adams and Bowman (2), however, have suggested that this "nonsedimentable" material might represent a smaller or less-dense infectious particle. Crispens (16) found that the "nonsedimentable" fraction was more resistant to heat inactivation in the presence of $MgCl_2$ than was the sedimentable fraction. The significance of these preliminary findings is uncertain, and further experiments must be performed to determine whether they represent part of a normal distribution curve, procedural artifacts [mechanical contamination or "fragmentation" of the virus (46*b*)], or a fundamental difference in the size, density, and properties of 0.1% of the particles.

INFECTIOUS NUCLEIC ACID

By employing the phenol-ether extraction method of Gierer and Schramm (22), an infectious ribonucleic acid (RNA) was recovered from LDV (42). The success with the phenol-ether method made it clear that the infectious nucleic acid from LDV was not adversely affected by exposure to ether. However, earlier experiments (40) showed that the infectivity of the intact virus was completely destroyed after treatment with ether. These findings suggested that ether might be inactivating the intact virus by altering its coat so as to expose the viral nucleic acid to the ribonuclease in the medium. Experiments in our laboratory (43) showed that this was the case (Table 5). When ribonuclease was removed from the virus pool, extraction with ether, butanol, chloroform, or phenol led to the recovery of an infectious nucleic acid, whereas in the presence of ribonuclease the intact virus was inactivated by both ether and butanol (43, 46*a*). Surprisingly, in the presence of plasma known to contain ribonuclease, chloroform and phenol yielded an infectious nucleic acid (42, 46*a*). The recovery of an infectious nucleic acid from a crude virus pool

TABLE 5. *Methods of obtaining an infectious nucleic acid from LDV*

Method of extraction	Recovery of an infectious nucleic acid	
	Partially purified virus (ribonuclease removed)	Crude virus (ribonuclease present)
Ether	Yes	No
Butanol	Yes	No
Chloroform	Yes	Yes
Phenol	Yes	Yes

(ribonuclease present) suggested that chloroform and phenol had removed or destroyed the ribonuclease; otherwise the nucleic acid would have been inactivated. In support of this argument are recent experiments which showed that the concentration of ribonuclease in the pool was substantially reduced after extraction with either chloroform or phenol (46*a*). The titer of the infectious nucleic acid obtained by the ether, butanol, chloroform, and phenol methods was quite similar, usually between $10^{1.5}$ and $10^{3.0}$ ID_{50} per milliliter. However, this represents a loss of over $10^{7.0}$ ID_{50} per milliliter, since the titer of the starting material was $10^{10.0}$ ID_{50} per milliliter. The low efficiency of infection of the nucleic acid or the removal of some of the original virus from the aqueous layer by the organic solvents (10, 30), or both, might be responsible for the low titer of the extracts. The fact that three different lipid solvents altered the coat of the virus so as to expose or release its nucleic acid suggests that the virus coat might contain a lipid.

Further evidence that LDV is a RNA virus comes from the chemical analysis on plasma pellets performed by Adams and Bowman (2). These

workers found that a pellet prepared from 100 ml of normal mouse plasma contained 40 μg of RNA, whereas a pellet prepared from 100 ml of viremic plasma contained 60 μg of RNA. They concluded that the difference was due to viral RNA. The base composition of the isolated RNA from the viral pellet was 22.0% adenine, 34.5% guanine, 23.5% cytidine, and 20.0% uridine. Almost identical figures were obtained from the RNA of the normal pellet (2).

GROWTH IN VIVO AND IN VITRO

The growth curve of LDV was studied by infecting mice intraperitoneally. At various intervals thereafter, the titer of the virus in the peripheral circulation was determined (40). Virus multiplication began within 6 hr, and an extremely high titer ($10^{10.8}$ ID_{50} per milliliter) was reached within 24 hr (Fig. 3). The titer dropped rapidly

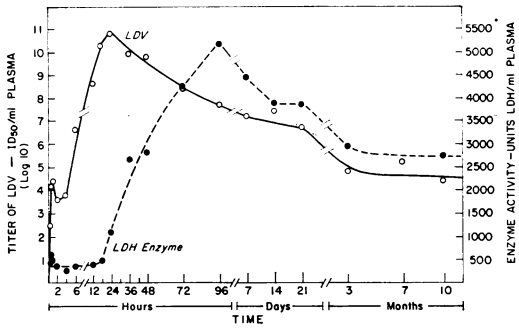

FIG. 3. *Growth curve of LDV and corresponding change in plasma LDH activity. Reprinted from Notkins and Shochat (40) by permission of The Rockefeller Institute Press.*

over the next 72 hr to $10^{7.8}$ ID_{50} per milliliter and finally leveled off at about $10^{5.0}$ ID_{50} per milliliter for the duration of the animal's life. Although the virus titer was highest at 24 hr, the plasma LDH activity was only slightly above normal. However, at 96 hr when the virus titer had decreased 1,000-fold, the enzyme activity was at its height (10- to 12-fold above normal). The enzyme activity then decreased and leveled off at five- to sevenfold above normal for life.

Yaffe (71) was the first to show that LDV could be propagated in vitro by use of primary cultures of mouse embryos. Although LDV failed to produce any cytopathology or enzyme elevation in vitro, the virus was detected by inoculating the tissue-culture supernatant fluids into recipient animals and testing for the characteristic 5- to 10-fold increase in plasma LDH. By serial weekly passage to primary mouse-embryo cultures, Yaffe found that the virus could be propagated indefinitely. Primary explants of mouse liver, spleen, and peritoneal macrophages have also been used to grow the virus (18, 48, 71). Evans (18) showed that virus multiplication began within 12 hr, reached a peak at about 24 hr, and slowly decreased until virus was no longer detected at 10 days. Similarly, Georgii et al. (21) found that the virus disappeared from the cultures within 10 days, although cultures prepared from embryos that had been infected in utero maintained the virus for a slightly longer time (16 days). Evans (18) found that virus replication was affected by the age of the culture. Cells that were in culture for 24 hr prior to inoculation yielded a higher virus titer than did cells that had been in culture for 7 days. Virus multiplication did not occur in 10- to 14-day-old cultures (18), secondary cultures, or infected primary cultures that were subcultured (71). Continuous cell lines (HeLa), rhesus monkey kidney cells, and mouse tumor cells also failed to support virus multiplication (18, 48, 71). The failure to propagate the virus longer than 2 weeks and the requirement for primary mouse cultures suggest that specific cells are required for virus replication and that these cells become altered or die after a prolonged stay in tissue culture. Virus titers greater than $10^{6.0}$ ID_{50} per milliliter have not been reported in vitro (18), whereas titers of $10^{10.5}$ ID_{50} per milliliter are routinely obtained in vivo (40).

METABOLIC AND MORPHOLOGICAL PATHOLOGY

Although LDV was first recognized by its ability to produce a 5- to 10-fold increase in plasma LDH, it soon became apparent that a number of other enzymes were elevated in the plasma of infected mice (31, 38, 39, 47). The increase in isocitric dehydrogenase (ICDH) almost equalled that of LDH, whereas malic dehydrogenase (MDH), phosphohexose isomerase, and glutamic oxaloacetic transaminase were elevated to a lesser extent (Fig. 4). As with LDH, these enzymes remained elevated for months (39). In addition, Plagemann et al. found that glutathione reductase was elevated (47). However, aldolase, alkaline and acid phosphatase, leucine amino peptidase, glucose-6-phosphate dehydrogenase, and α-glycerophosphate dehydrogenase were not affected by the virus (39, 47).

The possibility that the increase in glycolytic enzyme activity in the plasma reflected a basic metabolic disorder on the cellular level led Riley to study the glycolysis of infected and uninfected tumors. He found that the anaerobic glycolysis of a methylcholanthrene-induced tumor which had been infected with LDV was higher than its uninfected counterpart (58). However, not all infected tumors showed an increase in glycolysis

(Riley, *personal communication*). Furthermore, the literature contains no information as to the effect of the virus on the metabolism of tissues from mice not bearing tumors. Thus, further experiments are needed to determine whether LDV has any effect on glycolysis.

A number of investigators suggested that the increase in plasma enzyme activity might be due to the release of intracellular enzymes from damaged cells. However, gross and microscopic examination of organs from infected animals failed to reveal any evidence of pathology (6, 55), except for splenomegaly (59) and slight lymph node enlargement (63). Initially, it was reported (59) that the spleens of infected mice weighed 100 to 200% more than those of normal mice, but subsequent experiments showed that this was probably due to the presence of *Eperythrozoon coccoides* (62, 63). Studies in our laboratory revealed that only 21 of 55 infected mice had spleens that weighed 30% more than normal. The splenomegaly occurred within 72 hr after injection of the virus and persisted for at least 1 month. In 1961, Pope (49, 50) reported the recovery from wild mice in Australia of a virus (WM1) that produced slight to moderate splenomegaly and general enlargement of lymph nodes in over 50% of the animals. A persistent viremia was also detected. Microscopically, the splenomegaly was due to hyperplasia of both red and white pulp, and the enlargement of the lymph nodes was due to medullary hyperplasia and an increase in the number of plasma cells. Recently, Pope and Rowe (51) showed that the WM1 virus and LDV are identical. Whether the splenomegaly following infection with LDV is a primary effect due to virus multiplication or a secondary effect remains to be determined. However, it is quite clear that the elevated enzymes in the plasma of infected animals did not come from the spleen, since splenectomized mice showed an even greater enzyme elevation after infection with LDV than did nonsplenectomized mice (57).

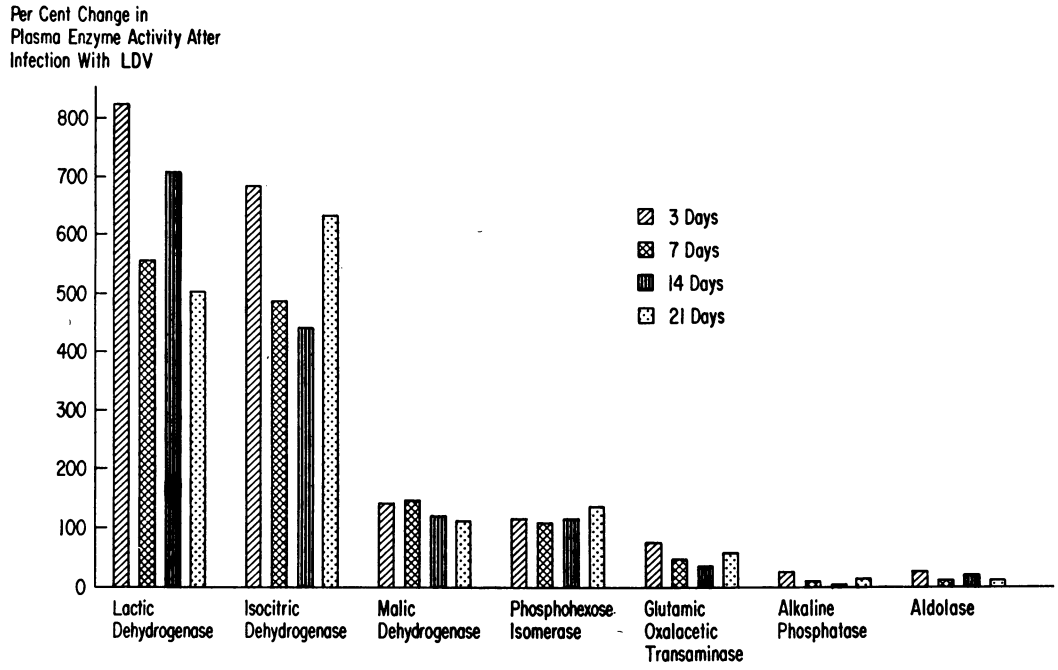

FIG. 4. *Per cent increase in the activity of various plasma enzymes after infection with LDV. Reprinted from Notkins et al. (39) by permission of The Rockefeller Institute Press.*

The possibility that the increase in plasma LDH might be due to destruction of red blood cells was studied by Riley (58). He noted that injection of LDV into splenectomized, irradiated, and tumor-bearing mice resulted in a severe anemia (57, 58). However, further experiments showed that the anemia was not due to LDV but to *E. coccoides* (62).

The failure to demonstrate morphological pathology (except slight splenomegaly and lymph node enlargement) suggested that the increase in plasma enzyme activity might be due to subtle alterations in the permeability of infected cells. Because it would be difficult to detect these changes by the usual gross and microscopic techniques, several investigators thought that com-

parison of the isozyme pattern of infected plasma with that of various organs might give a clue as to the origin of the elevated plasma enzymes (48, 66*b*, 68). Starch-gel electrophoresis showed that the increase in plasma LDH after infection with LDV was due largely to the increase in the slowest-migrating component, which Plagemann (48) referred to as LDH_1. $LDH_{2,3,4}$ were elevated to a lesser extent. Since LDH_1 was found in every organ examined and $LDH_{2\,3,4,5}$, were found in many of the organs, the isozyme pattern of infected plasma was not sufficiently characteristic of any one tissue to give a definitive answer regarding the origin of the elevated plasma enzymes.

As an alternative to the cell permeability hypothesis, it was suggested that the increase in plasma enzyme activity might be due to de novo enzyme synthesis. Studies from three laboratories

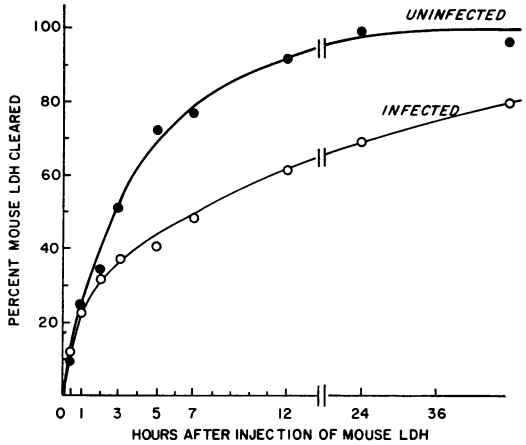

FIG. 5. *Clearance of 20,000 units of LDH (mouse liver) by infected and uninfected mice (45).*

(5, 47, 68), however, failed to reveal any significant differences in the enzyme content of tissues from infected and uninfected animals.

MECHANISM OF ENZYME ELEVATION

Although leakage of enzymes from damaged cells and de novo enzyme synthesis have been suggested as possible explanations for the increase in plasma enzyme activity after infection with LDV, experiments from several laboratories, as indicated above, failed to provide support for these hypotheses. The demonstration that LDV could replicate in vitro without producing any enzyme elevation (18, 21, 71) suggested another possibility, that the increase in plasma LDH might be an in vivo phenomenon due to impaired clearance of endogenous enzymes from the peripheral circulation. To test this hypothesis, LDH was injected intravenously into normal and infected mice, and the disappearance of enzyme activity (clearance) from the peripheral circulation was studied (45). Infected mice cleared intravenously administered LDH (homologous) at a slower rate than did normal mice (Fig. 5). Within 7 hr, normal mice had cleared over 75% of the

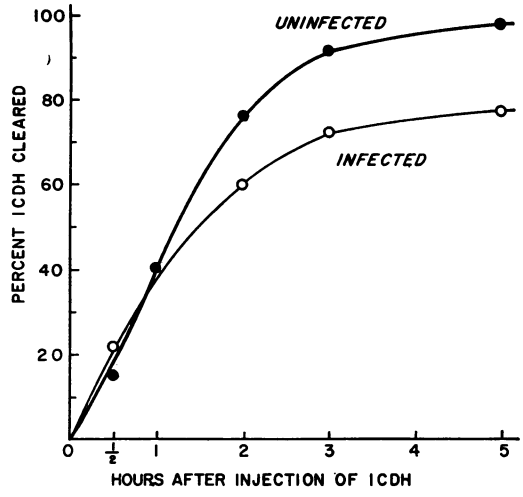

FIG. 6. *Clearance of 750 units of ICDH (pig heart) by infected and uninfected mice (45).*

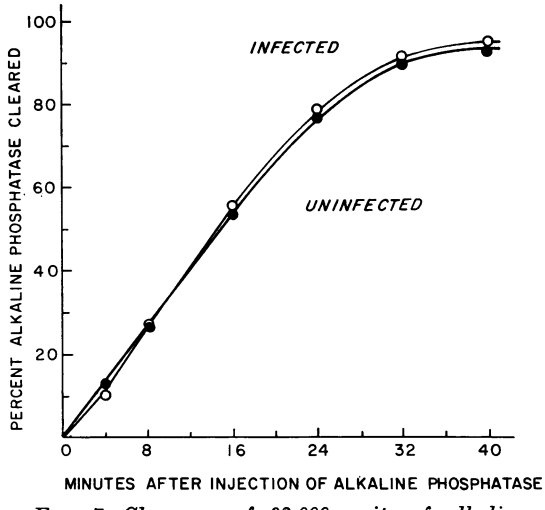

FIG. 7. *Clearance of 23,000 units of alkaline phosphatase (calf intestine) by infected and uninfected mice (45).*

injected enzyme, whereas it took infected mice almost 48 hr to clear the same amount of enzyme. ICDH (heterologous) was also cleared more slowly by infected animals (Fig. 6), whereas alkaline phosphatase (heterologous), an enzyme which was not elevated after infection with LDV, was cleared at the same rate in normal and infected animals (Fig. 7). In addition, Bailey (6*a*)

found that malic dehydrogenase and glutamic oxaloacetic transaminase were cleared more slowly by infected animals. These findings suggested that any disease state, infection, or chemical that damaged cells and released endogenous enzymes into the peripheral circulation would result in a more prolonged enzyme elevation in LDV-infected animals than in normal animals. Because of the slower rate of clearance, the enzyme elevation would also appear to be higher in proximately the same rate as uninfected mice not bearing tumors, there is considerable evidence in the literature which indicates that the amount of enzyme entering the circulation of tumor-bearing mice is greater than that of mice not bearing tumors (12, 27, 70). Infection of tumor-bearing mice with LDV would thus decrease the rate of enzyme clearance and lead to the accumulation of endogenous enzymes. The higher enzyme influx into the circulation of tumor-bear-

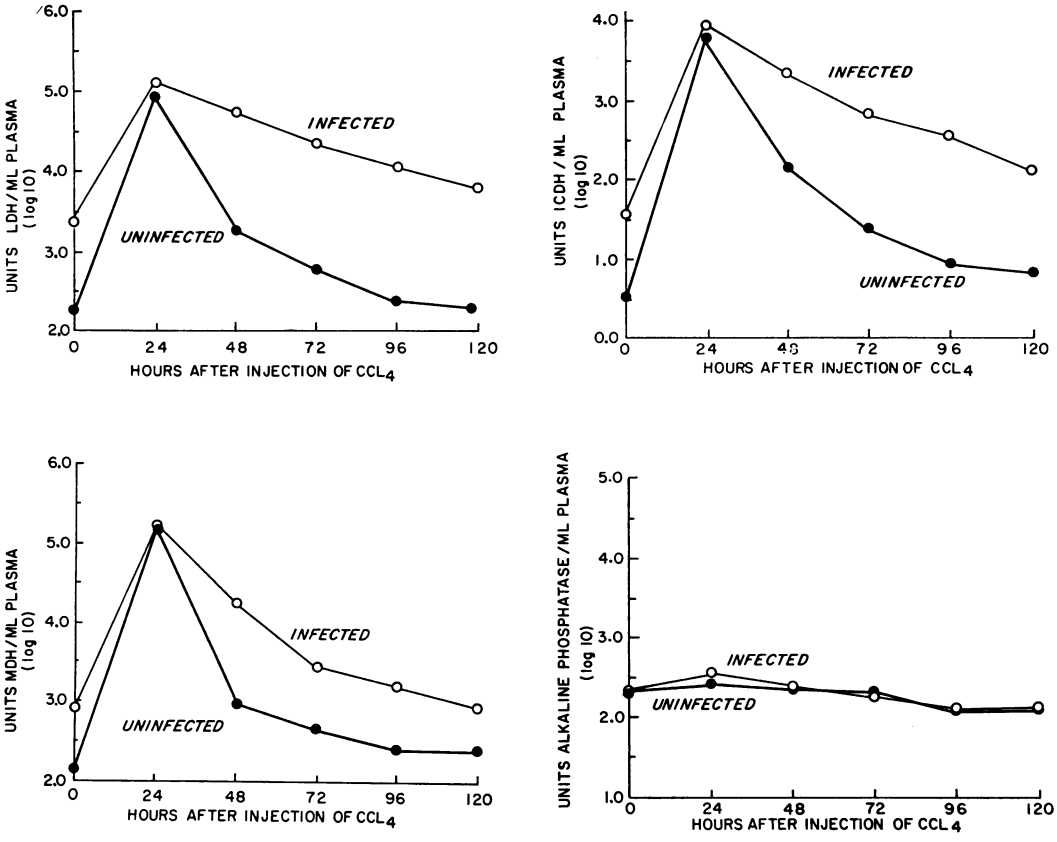

FIG. 8. *Enzyme activity in the plasma of normal and infected mice after intraperitoneal injection of 0.001 ml of carbon tetrachloride (45).*

infected animals. As shown in Fig. 8, carbon tetrachloride produced extremely high levels of LDH, ICDH, and MDH in both normal and infected animals (45). In normal animals, however, the enzyme level fell rapidly toward the base line, whereas in infected animals the return to the base line was much slower and substantial enzyme elevation persisted for several days. Similarly, the enzyme elevation in the plasma of tumor-bearing mice after infection with LDV (39) could be explained on the basis of impaired enzyme clearance Although preliminary studies in our laboratory indicated that uninfected tumor-bearing mice clear intravenously administered LDH at aping mice would, therefore, result in a far greater accumulation of enzymes after infection with LDV than would occur in mice not bearing tumors. The data in Table 6 support the contention that the height of the enzyme elevation in normal and tumor-bearing mice after infection with LDV is related to the extent of the enzyme influx. In normal mice with a plasma LDH of less than 1,000 units per ml, the increase in LDH activity 48 hr after injection of the virus was 1,600 units per ml. In tumor-bearing mice with a plasma LDH of approximately 2,000 and 5,000 units per ml, the increae in LDH activity 48 hr after injection of the virus was 8,000 and 19,000

units per ml, respectively. In other experiments, it was shown that the number of units of enzyme remaining in the circulation at a given time after injection of different doses of LDH (ranging from 12,500 to 75,000 units per ml) was directly proportional to the amount of enzyme injected (45).

The level of LDH in the peripheral circulation is, thus, dependent upon two factors: the rate of influx and the rate of clearance. Within 13 hr after injection of LDV, infected mice were no longer able to clear enzyme at the normal rate (45). Mahy (33) found that the impairment was greatest at 48 hr after injection of the virus; at that time, it took infected mice seven to eight times as long as normal mice to clear intravenously administered LDH. Thus, the time lag between the onset of impaired enzyme clearance (13 hr postinfection) and maximal enzyme elevation (96 hr postinfection, Fig. 3) appears to represent the time required for normally released endogenous enzymes to accumulate in the blood. Although impaired enzyme clearance could be demonstrated for months (45), the extent of the impairment was not quite as great as during the acute phase of the infection (33). The persistent enzyme elevation in the chronically infected animal thus appears to be due to the establishment of a new equilibrium between the normal influx of endogenous enzymes and impaired enzyme clearance. Although there is no evidence at the present time that an increase in enzyme influx contributes to the enzyme elevation in the plasma of infected animals, this possibility has not been excluded.

TABLE 6. *Relationship of initial enzyme level in the plasma of tumor-bearing mice to LDH elevation after infection with LDV**

Group†	Initial level of LDH	Increase in LDH at 48 hr
	units/ml	*units/ml*
Controls		
Mice without tumors, normal plasma LDH.....	825	0
Tumor-bearing mice, low plasma LDH.....	2,478	1,958
Tumor-bearing mice, high plasma LDH.....	5,522	4,962
Infected		
Mice without tumors, normal plasma LDH.....	831	1,650
Tumor-bearing mice, low plasma LDH.....	1,965	8,175
Tumor-bearing mice, high plasma LDH.....	4,963	19,250

* Data from Notkins and Goffman.

† Mice bearing plasma cell tumor 70429 were divided into two groups on the basis of the LDH activity in their plasma (low and high). One half of each group was kept as controls, and the other half was injected with LDV. The increase in plasma LDH was determined 48 hr later. Each figure represents the average of 10 animals.

The mechanism by which infection with LDV leads to impaired clearance of enzymes remains to be elucidated. This problem is complicated by the fact that the mechanism of clearance of LDH in normal animals is not known. Since the reticuloendothelial system (RES) has been implicated in the clearance of a number of substances, it was pertinent to see whether infection with LDV produced any loss in the functional capacity of this system as measured by the rate of clearance of colloidal carbon particles (45). Impairment of the RES occurred within 18 hr after injection of LDV (Fig. 9). However, at the end of 1 week, the capacity of the RES to clear carbon particles had returned to normal (45). Almost identical results were obtained by Mahy (33). Since carbon clearance had returned to normal within 1 week, whereas LDH clearance remained impaired for months, it was not possible by this technique to implicate the RES in impaired enzyme clearance. However, since carbon clearance and enzyme clearance may be handled in very different ways and even by different cells, involvement of the RES has not been ruled out. In fact, evidence is discussed below which suggests that the RES does play a role in the clearance of LDH. However, a number of other factors secondary to the virus infection, such as alterations in blood flow and volume, enzyme binding and

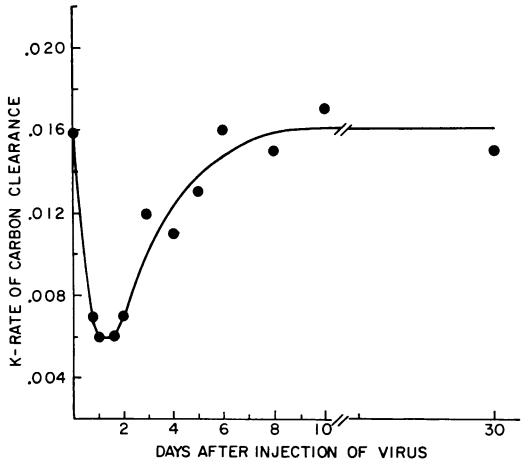

FIG. 9. *Rate of clearance of colloidal carbon particles (16 mg/100 g of body weight) from the peripheral circulation at different times after injection of LDV (45).*

stability, competitive inhibition, and hormones, could affect the rate of disappearance of enzyme activity from the peripheral circulation; these possibilities remain to be investigated.

EPIDEMIOLOGY

LDV has not been recovered from any species except the mouse (48, 49, 59). Inoculation of the virus into rats, hamsters, guinea pigs, and rabbits failed to raise the plasma LDH of these animals, and attempts to demonstrate the presence of the virus by injecting the plasma of these animals back into normal mice was unsuccessful (Notkins, *unpublished data*). Mice of all strains appear to be susceptible to the virus (59). Except for two reports (48, 59) of infected colonies with no known previous exposure to LDV, the virus is rarely found among normal laboratory mice. However, LDV has been found in wild mice in Maryland, Australia, and England (49, 51, 65*a*). Pope and Rowe (51) suggested that wild mice may serve as an important virus reservoir, and may be responsible for the occasional introduction of the virus into the laboratory.

Mice were successfully infected with LDV by the intravenous, intraperitoneal, intramuscular, intracerebral, and subcutaneous routes (59). In our laboratory, dermal application of the virus failed to infect mice unless there were breaks in the skin, and the oral route was successful only if a high concentration of virus was used (over $10^{6.4}$ ID_{50} per milliliter; 41). These findings suggested that if LDV were present in high concentration in the excretions of infected mice, transmission to uninfected cage mates could occur by the oral route, or by the dermal route if there were breaks in the skin. Examination of the excretions of infected animals (41) showed that the virus was present in the urine, feces, saliva, and milk (Table 7). As in the blood, the virus titer was highest at 24 hr postinjection and decreased during the following weeks. At the end of 16 and 28 days, respectively, virus was no longer detected in the urine and saliva, although it was present in the feces for at least 135 days (41). Despite the presence of virus in the excretions of these animals, the transmission rate of LDV from infected to uninfected mice housed together for 135 days was less than 5% (40). The low rate of transmission was not surprising, however, because the concentration of virus in the urine, feces, and saliva was relatively low as compared with the high concentration of virus required to infect animals by the oral route (over $10^{6.4}$ ID_{50} per milliliter; 41). In contrast to the low rate of transmission in our laboratory, high or variable rates were reported by other investigators (15, 48). Because animals of different ages, sex, and strains were used, it was pertinent to see whether these factors affected the rate of transmission of LDV. Our studies (44) revealed that the transmission rate was low among CAF-1, C3H/Hen, Balb/c, and C57 Black mice. A high rate of transmission, however, was found among General Purpose (GP) Swiss mice. Further observations revealed extensive bite marks and scars over the bodies and tails of the GP mice, whereas relatively few such scars were found among the other strains (44). These scars suggested that the GP mice were transmitting the virus by biting and inoculating infected saliva. Since young mice did not bite their cage mates as frequently as did older mice, and since female mice fought less than males, it was thought that the transmission rate of LDV would be lower in these groups. The data in Table 8 show this to be the case, for while 100% of the older males became infected only 3% of the young females became infected. Further support for the argument that LDV was transmitted by the inoculation of infected saliva through biting came from experiments in which biting was prevented by removing the incisors (44). In these experiments, the rate of transmis-

TABLE 7. *Titer of LDV in the urine, feces, saliva, and milk*

Time after injection of LDV	ID_{50}/ml (log 10)			
	Urine*	Feces*	Saliva*	Milk†
days				
1	4.3	5.9	4.2	7.2
2	3.3	4.3	3.5	6.8
3	3.3	4.7	2.8	5.8
9	3.1	4.0	2.4	4.4
16	<2.5‡	3.8	2.8	4.2
28	<2.5‡	4.3	<2.0‡	—
42	<2.5‡	5.0	<2.0‡	—
60	—	3.9	—	—
135	—	2.5	—	—

* Data from Notkins and Scheele (41).
† Data from Notkins and Feller.
‡ Virus not detected.

TABLE 8. *Effect of sex and age on the transmission of LDV in GP Swiss mice (44)*

Sex	Age	Transmission rate*
		%
Male	Over 6 months	100
Female	Over 6 months	57
Male	Under 6 weeks	31
Female	Under 6 weeks	3

* Infected and uninfected mice were housed in the same cage, and at the end of 1 week the number of newly infected mice was determined.

TABLE 9. *Transmission of LDV to offspring*[a]

Time of infection of mothers	No. of litters with infected progeny/total no. of litters	No. of progeny infected[b]/total no. of progeny	Progeny infected
			%
Prior to mating[c]	1/14	1/108	0.9
During gestation[d]	17/17	62/68	91.2
Postpartum[e]	14/14	51/99	51.5

[a] Reprinted from Notkins and Scheele (41) by permission of The Rockefeller Institute Press.
[b] The number of progeny infected was determined when the animals were 2 to 3 weeks old.
[c] Infected 1 week prior to mating.
[d] Infected between the 7th and 18th day of gestation.
[e] Infected within 48 hr after giving birth.

sion dropped from 100% in normal mice (incisors present) to 5% in partially edentulous mice (incisors removed). In addition to transmission by inoculation of infected saliva, ingestion of tissues from acutely infected animals as a result of cannibalism also led to the transmission of the virus (44). Thus, the transmission of LDV depends on the titer of the virus in the inoculum and on the route of inoculation. The age, sex, and strain of the animals affect the rate of transmission in that these factors influence the biting habits of the animals.

The demonstration that mice could be infected by the oral route if exposed to over $10^{6.4}$ ID_{50} per milliliter, and that there were at least $10^{7.0}$ ID_{50} per milliliter in the milk of acutely infected animals, suggested that LDV could be transmitted from the acutely infected mother to her offspring by nursing. When the mothers were infected with LDV within 48 hr after giving birth, so that the virus titer in the milk during the acute phase was $10^{7.0}$ ID_{50} per milliliter, 51.5% of the sucklings became infected (Table 9; 41). However, when the mothers were infected 1 week prior to mating, so that the virus titer in the milk was less than $10^{4.4}$ ID_{50} per milliliter during nursing, only 0.9% of the offspring became infected. These experiments suggested that the transmission of LDV was dependent at least in part on the titer of the virus in the milk. They also showed that the virus was not transmitted via the placenta in the chronically infected animal. However, if mothers were infected during gestation rather than prior to mating, over 91% of the offspring became infected (Table 9), suggesting that the virus had crossed the placenta. These observations were substantiated by Georgii et al. (21), who showed that within 72 hr after inoculating pregnant mice with LDV the virus could be recovered from the embryos. The transmission of LDV across the placenta in the acutely infected animal and the lack of transmission in the chronically infected animal appear to be related to the titer of the virus in the maternal circulation. That is, in the acutely infected animal the virus titer reaches $10^{10.5}$ ID_{50} per milliliter within 24 hr after injection, whereas, in the chronically infected animal, the titer is between $10^{5.0}$ and $10^{7.0}$ ID_{50} per milliliter throughout the gestation period. If a high virus titer is required to infect the fetus by the placental route, infection of the fetus is more likely to occur in the acutely infected animal than in the chronically infected animal. The role that antibody may play in protecting the fetus of the chronically infected mother against the virus is unknown, because evidence for antibody against LDV is still lacking.

RELATIONSHIP TO TUMORS AND VIRUSES

Since the initial report on the association of LDV with mouse tumors (55), over 50 types of tumors from laboratories throughout the world were found to be contaminated with this virus (Table 10). Tumors that had been transplanted for many generations were more often contaminated than primary tumors (1, 36, 37, 47, 71) or tumors that had been transplanted for only a few generations. Tumors that were free from the virus in one laboratory were frequently found contaminated in other laboratories. Because of the constant viremia, tissues removed at any time during the life of the infected animal would contain the virus. Thus, LDV could be transmitted indefinitely by serial passage of infected tissues, such as tumors. Once LDV is introduced into a particular laboratory, transmission to uninfected cage mates and accidental contamination of parenterally administered materials could easily lead to the wisespread dissemination of the virus and might account for the large number of contaminated tumors. However, tumors and viruses can be freed from LDV by passage through rats (37) or through cell lines which do not support LDV replication (1, 47). Thus, sarcoma 180, Moloney virus, Friend virus, and pneumonia virus of mice originally contaminated with LDV were freed from LDV by passage through tissue culture or rats (1, 32, 37, 47, 66*a*).

Nontumor viruses are also contaminated with LDV (Table 10). Wenner et al. (69) suggested that a number of viruses, in addition to LDV, could produce an increase in plasma LDH, but no effort was made to determine whether these viruses were contaminated with LDV. Other investigators (1, 20, 32, 37, 48, 71) showed that in the absence of LDV most of the viruses they

TABLE 10. *Tumors and viruses contaminated with LDV*[a]

Tumors contaminated with LDV		
Transplanted	Sarcoma 1137, solid and ascitic (52)	Leukemia L-1210 (57)
Ehrlich carcinoma, solid and ascitic (57, 71, 48, 20)	Ascites tumor SA1 (20)	Leukemia L-4946 (57)
Nelson ascites (57)	SBL1/p sarcoma (71)	Friend Leukemia (57)
Carcinoma 1025 (57)	Sarcoma MC48 (36)	Rauscher leukemia (57)
Bashford carcinoma 63 (57)	CD/5 lymphoma (5)	Moloney leukemia (37, 57)
Lewis bladder carcinoma (57)	Mecca lymphosarcoma (57)	EL 4 leukemia (57)
Lewis lung carcinoma (57)	Lymphosarcoma P 1798 (57)	C 1498 leukemia (57)
Carcinoma 755 (57)	Lymphosarcoma 6C3HED (48)	P 1534 leukemia (68)
ELD ascites carcinoma (12)	Ridgway osteogenic sarcoma (57)	Cloudman S91 melanoma (57)
Miyono adenocarcinoma (57)	Wagner osteogenic sarcoma (57)	Harding-Passey melanoma (57)
Mammary adenocarcinoma EO-771 (57)	Rhabdomyosarcoma (BW-10139) (57)	B-16 melanoma (57)
C3HBA mammary adenocarcinoma (57)	Fibrosarcoma (SA/γ) (36)	Glioma 26 (57)
DBA-59 mammary carcinoma (5)	Reticulum cell sarcoma C57BRT (15)	Adrenotropic At T20 (57)
Mammary carcinoma (12)	Mammary tumor TA 3 (48)	Plasma cell neoplasm 5647 (57)
Sarcoma 180, solid and ascitic (48, 57, 1)	L5178Y ascites tumor (48)	Ovarian cell granulosa tumor (H4929) (57)
Sarcoma 37 (36, 1)	Hauschka-Klein ascites tumor (48)	
Sarcoma T-241 (57)	Hepatoma 129 (48)	*Spontaneous*
Sarcoma MA 387 (57)	Hepatoma 134 (48, 71)	Adenocarcinomas mammary (55)
Tumors not contaminated with LDV		
Transplanted	Pituitary thyrotropic tumor (Furth-Money) (57)	Methylcholanthrene-induced tumors (57)
Plasma cell tumor 70429 (37)	Hemangioendothelioma (BW-6473) (57)	Dibenzanthracene-induced tumors (57)
Reticulum cell sarcoma H6867 (37)	Hepatoma (BW-7756) (57)	
Mammary carcinoma C3HBA (37)	Ovarian granulosa cell tumor (E-11731) (57)	*Spontaneous*
Melanoma DM1 (37)		Mammary tumors (36, 71, 37, 57)
Lymphomas (71)	*Induced*	AKR Thymoma (57)
Sarcomas induced by benzpyrene (71)	Stilboestrol-induced testicular tumors (37)	C57L/J Thymoma (57)
Sarcoma 180 (47)	MT1 Sarcoma induced by polyoma (71)	AKR Leukemia (1, 36, 71)
Sarcoma B/BP (36)	Thymic lymphoma induced by C57BL leukemia virus (71)	Others (20, 71)
Mammary carcinoma (i/36) (36)	Radiation induced leukemias (57, 71)	

Viruses contaminated with LDV	Viruses not contaminated with LDV	
Moloney (1, 71, 20)	Moloney (1, 37)	Newcastle disease[b]
Friend (32)	Friend (32)	Mouse hepatitis (Nelson's PR-1)[b]
Rausher[b]	Gross (20)	Mouse hepatitis (Manaker's A 59)[b]
West Nile[c]	Polyoma (71, 1, 32, 20, 48)	Vaccinia (32)
Mouse hepatitis (Manaker's A 59) (37)	Toolan[c]	Lymphocytic choriomeningitis (32)
Spleen weight increase factor (66)	C57BL-agent (71)	Encephalomyocarditis (32)
Pneumonia virus of mice (66*a*)	AK virus (71)	Poliomyelitis (20)
Eperythrozoon coccoides[d] (62)	Influenza A[b] (32, 20)	

[a] Contamination of a particular tumor or virus in one laboratory does not mean that strains in other laboratories are contaminated.
[b] Notkins, *unpublished data.*
[c] Riley, *personal communication.*
[d] *Eperythrozoon coccoides* is not a virus but belongs to the order Rickettsiales.

tested failed to increase plasma LDH. However, it was found that encephalomyocarditis virus (32), ectromelia virus (20), and *E. coccoides* (4, 62, 63) caused cell damage and released intracellular enzymes into the circulation. The particular enzymes released and the magnitude and duration of the enzyme elevation was quite different than with LDV. Thus, if the inoculation of a particular virus preparation results in an increase in plasma LDH, it must be ascertained whether the virus under question or contamination with LDV is responsible for the enzyme elevation. In the absence of a specific serological

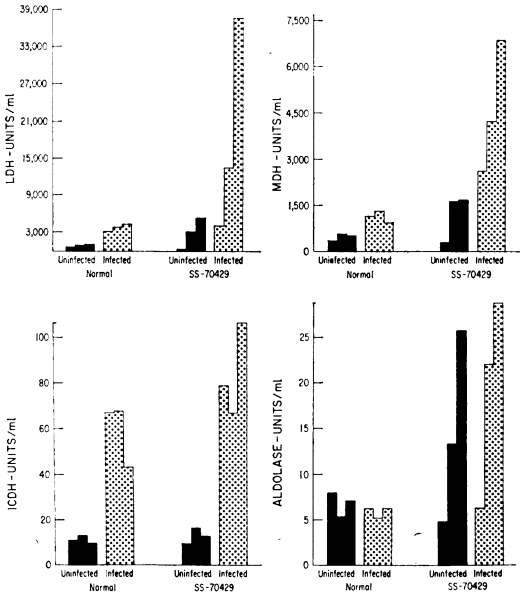

FIG. 10. *Activity of enzymes in the plasma of normal and tumor-bearing mice after infection with LDV. The bars within each group (left to right) represent the 3rd, 10th, and 17th days postinjection (39).*

test for LDV, the characteristic enzyme elevation, the extremely high virus titer at 24 hr, and the chronic viremia are sufficiently unique to serve as good identifying markers to differentiate LDV from other known viruses (51).

Although there have been reports of a close correlation between the growth of certain murine tumors and the increase in plasma LDH (26, 54), other studies showed that the enzyme level in the plasma was substantially elevated prior to the gross appearance of the tumor (26, 34). Since these experiments had been performed before it was recognized that many tumors were contaminated with LDV, the relationship between tumor growth and plasma enzyme elevation was reinvestigated in the presence and absence of the virus (1, 38, 39, 47, 56, 71). Uninfected tumor-bearing mice showed no increase in plasma enzyme activity at the end of 3 days, whereas infected tumor-bearing mice showed increases in LDH, MDH, and ICDH which were equivalent to the increases seen in the plasma of infected mice not bearing tumors (Table 10). Aldolase, which is not elevated by LDV, was the same in both groups. Thus, the early rise in plasma enzyme activity which occurred prior to the gross appearance of the tumor was not due to the tumor, but to LDV. In the absence of LDV, the activity of the enzymes in the plasma of tumor-bearing mice remained within the normal range until the tumor became palpable, and then the enzyme elevation roughly paralleled the growth of the tumor. Figure 10 shows that the activity of LDH in the plasma of infected tumor-bearing animals on day 17 (38,000 units per ml) was not only higher than that of uninfected tumor-bearing animals (5,500 units per ml) but was several times greater than that which would occur by simply adding the plasma LDH of uninfected tumor-bearing animals (5,500 units per ml) to that of infected animals not bearing tumors (4,000 units per ml). Because, as indicated earlier, the amount of enzyme entering the circulation of tumor-bearing animals is greater than that of normal animals, infection of tumor-bearing animals with LDV would impede the clearance of this additional enzyme and result in a more than additive increase in plasma enzyme activity (synergism). The data in Table 6 show that the extent of the synergism was related to the amount of enzyme in the peripheral circulation prior to infection. Similarly, as shown in Fig. 10, the high level of LDH (eight- to ninefold above normal) in the uninfected tumor-bearing animal led to considerable synergism in the infected tumor-bearing animal. Conversely, due to the low levels of ICDH and MDH (zero to threefold above normal) in the uninfected tumor-bearing animal, infection with LDV resulted in little if any synergism. From these studies it can be seen that much of the increase in plasma enzyme activity which had previously been attributed to tumor growth was in fact due to contamination with LDV.

The effect of LDV on tumor growth was studied by Riley (56, 58). He found that neither the time of appearance nor the growth rate of a primary tumor induced by methylcholanthrene was influenced by LDV. However, after transplantation, the infected tumor grew more rapidly and reached a greater size than its uninfected counterpart. The significance of these findings is difficult to interpret, because in other experiments the growth rate of infected tumors was unaltered, or was inhibited (58). Other laboratories (1, 38, 47,

71) have failed thus far to report any difference in the growth rate of infected versus uninfected tumors.

The effects that unsuspected contamination with LDV may have had on other viruses are just becoming apparent. For instance, three laboratories recently attempted to devise a rapid assay for the Moloney leukemia virus by employing viral interference. They found that the materials containing the Moloney virus exerted marked interference on vesicular stomatitis (64), influenza (39), and vaccinia (3) viruses, but further studies revealed that it was not the Moloney virus but LDV which was responsible for the interference. Baron (7) showed that animals infected with LDV had low levels of interferon in their circulation at 24 and 48 hr after injection of the virus, but not thereafter. Whether these low levels of interferon play any role in viral interference has not yet been determined.

Contamination with LDV might also affect the results and the interpretations of experiments through its action on the RES. The impaired clearance of carbon particles suggests that the rate at which other materials are cleared from the peripheral circulation of infected animals might also be impaired. If this proves to be the case, animals infected with LDV might be more susceptible to bacteria, viruses, tumors, drugs, chemicals, toxins, etc., than are normal animals. In this connection, the synergism between LDV and *E. coccoides* is pertinent (63). Infection of nonsplenectomized mice with *E. coccoides* resulted in a lowering of the hematocrit and an increase in white count, spleen size, and plasma LDH. In the presence of LDV, all of these conditions were exaggerated. The explanation for this synergism might lie in the fact that the impairment of the function capacity of the RES by LDV decreased the rate at which *E. coccoides* was cleared from the circulation. The increase in the titer of *E. coccoides* after infection with LDV (63) might thus be responsible for the exaggerated pathology. The enzyme synergism, as indicated above, can be explained on the basis of impaired clearance of enzymes.

From these studies, it can be seen that the widespread contamination of tumors, viruses, and animals with LDV adds considerably to the already complex problem of extraneous mouse viruses (64) and of the interpretation of experiments in which LDV is a contaminant.

DISCUSSION

LDV has proved to be a particularly interesting virus because of its unusual manifestations and wide distribution. The studies described in this review point to the possibility that some of the unusual manifestations of this virus, such as the persistent enzyme elevation, the high virus titer, the chronic viremia, and the lack of antibody, might be related to the effect of the virus on the RES. First, there is preliminary evidence which suggests that LDV is growing in the cells of the RES. Tissue-culture experiments showed that LDV replicates in primary explants which are rich in RES cells, such as spleen, liver, and peritoneal macrophages (18, 48, 71). Electron microscopy of peritoneal macrophages revealed virus particles within these cells (17*a*), but it still remains to be determined whether the virus is actually replicating in the macrophages or is simply being phagocytized. In addition, evidence is beginning to accumulate which suggests that the RES may play a role in the clearance of enzymes. A number of reports in the literature indicate that at least some protein is removed from the circulation by the RES (8, 13, 14, 24), and Fleisher and Wakim (19, 67) and Mahy (*personal communication*) have shown that blockade of the RES decreases the rate at which LDH is cleared from the peripheral circulation. These studies, if substantiated, would support the hypothesis that the lifelong enzyme elevation following infection with LDV is due to the virus growing in or damaging the very cells which remove endogenous enzymes from the circulation.

This raises the question as to what factors are responsible for the chronic nature of the infection. As indicated above, the virus appears to be growing in or damaging the RES cells which ordinarily play a major role in the defense of the host. Impairment of the functional capacity of this system might decrease the rate at which LDV is cleared from the peripheral circulation. Mims (35) showed that the titer of Semliki forest virus was substantially increased in the plasma of mice after blockade of the RES with thorotrast, and recently Mahy (*personal communication*) showed that the titer of LDV was also increased after thorotrast. Thus, the impairment of the RES at 24 hr after injection of LDV might be responsible for the very high virus titer of $10^{10.5}$ ID_{50} per milliliter. As the functional capacity of the RES returns to normal (as measured by carbon clearance), the titer of the virus in the blood decreases to a steady state of $10^{4.0}$ to $10^{6.0}$ ID_{50} per milliliter, which persists for the life of the animal. The steady state indicates that the virus is entering the circulation at the same rate at which it is being removed. However, until quantitative data on the clearance of LDV in normal and infected mice are obtained, the role that the RES plays in maintaining the chronic viremia cannot be properly evaluated.

Another factor that might be responsible for

the chronic viremia is the apparent lack of neutralizing antibody to LDV in mice. Although extensive studies have not been carried out, a number of investigators (Plagemann, Rowe, Du Buy, and Riley, *personal communications*) have attempted without success to obtain neutralizing antibody against LDV in species other than mice. If the outer coat of the virus contains lipid, as suggested earlier, this might make it a poor antigen and could account for the failure to obtain neutralizing antibody. On the other hand, the constant outpouring of viral antigens in the chronically infected mouse might produce a state of immunological unresponsiveness. Since macrophages are thought to play a role in initiating antibody production (13), the presence of the virus in these cells might adversely affect the immunological response of the host to LDV. Thus, the poor antibody response, the high virus titer, the chronic viremia, and the persistent enzyme elevation might all be due to the effect of the virus on the RES.

Although there are a number of viruses which, in vivo and in vitro, cause cell damage and release intracellular enzymes (20, 23, 28, 29, 32), LDV is the first virus which has been shown to increase enzyme activity in the peripheral circulation of an animal by decreasing the rate of enzyme clearance. Preliminary evidence suggests that LDV impairs the clearance of enzymes by its action on the RES; however, other target cells and factors which affect enzyme clearance (such as blood flow, enzyme binding and stability, hormones, etc.) remain to be investigated. LDV may thus serve as a useful tool in studying the mechanism of enzyme clearance. In the future, it will be of interest to see whether infection with LDV decreases the rate at which nonenzymatic proteins are cleared from the peripheral circulation and whether other viruses and disease states which act on the RES affect the rate of enzyme clearance.

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