SOME PROPERTIES OF THE LACTIC DEHYDROGENASE AGENT OF MICE

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Evidence for the viral nature of the lactic dehydrogenase (LDH) agent, discovered in the plasma of many tumor-bearing mice by Riley (1), has been obtained by several laboratories (2, 3). The ability of this agent to cause an increased activity of a number of enzymes in mouse plasma (4), persisting during the lifetime of the host (1) without causing apparent cellular damage merits further study.

This paper will present data on maintenance, titration, and partial purification of the LDH agent, its resistance toward organic solvents, its morphology and size as determined by electron microscopy, its activity as an interferon producer, and its lack of antigenicity.

Materials and Methods

Strain of LDH Agent.—The strain was obtained from a naturally infected Ehrlich ascites carcinoma. This Ehrlich ascites strain had been maintained for 2 years in CDF_1 mice (NIH strain).

Infectivity Titer.—Infectivity of the agent was assayed by injecting 0.1 ml of 10-fold serial dilutions, made in Eagle's medium plus 20 per cent veal infusion broth (5), into NIH G-P Swiss (20 to 25 gm) mice, 5 mice per dilution. Four to 5 days after inoculation blood was collected by orbital bleeding (6) with capillary pipettes, wetted with a heparin solution containing 1000 U.S.P. units heparin per ml, and pooled. LDH activity was determined on the plasma obtained from each pool by a modification of the method of Wroblewski and La Due (7). The number of infected mice at each dilution was estimated from a scale representing the stepwise increase of LDH activity contributed by each additional infected mouse (*vide infra*) and the results calculated by the Karber method. The titer was expressed as the negative $\log_{10}$ dilution of the sample which induced an increased lactic dehydrogenase activity in 50 per cent of the mice (ID_{50} per ml).

Preparation of LDH Agent.—Blood plasma or ascitic fluid from Ehrlich ascitic carcinoma-bearing mice (*vide infra*), infected 24 hours previously with the LDH agent, was harvested and centrifuged at 900 RCF for 10 minutes. The supernatant was diluted with an equal volume of 0.3 M potassium citrate and 0.003 per cent hyaluronidase, and digested at room temperature for 1 hour (8). It was centrifuged at 4000 RCF for 20 minutes. The supernatant was de-

canted and recentrifuged at 10,000 RCF for 6 minutes. The resulting supernatant was again decanted and centrifuged at 105,000 RCF for 2 hours in a Spinco model L ultracentrifuge. All centrifugations were done at 0 to 4° C. The pellet was resuspended in distilled water, in one-tenth of the original volume. Material prepared in this manner was used for the experiments presented below, unless otherwise indicated. If necessary, the agent, resuspended in distilled water, was stored at −25°C.

Electron Microscopic Examination.—Ultracentrifuged pellets of partially purified LDH agent were fixed in 1 per cent osmium tetroxide (buffered to pH 7.0), dehydrated, and embedded in maraglas (9). Sections were stained with lead citrate (10), and examined in an RCA EMU 3 E electron microscope.

TABLE I *a*

*Determination of the Infectivity Titer by Pooling the Plasma of 5 Mice**

No. mice infected	LDH units per ml plasma		
	Expected	SD	Class
0/5	740	108	0–1000
1/5	1618	157	1100–2000
2/5	2495	194	2100–2900
3/5	3373	225	3000–3700
4/5	4250	252	3800–4600
5/5	5128	277	4700–

* The scale representing the stepwise increase in LDH-activity contributed by each additional infected mouse is based on the LDH-activity of 67 control mice and 68 infected mice (see text).

RESULTS

Determination of the Infectivity Titer by Pooling the Plasma of 5 Mice.—A shortened procedure for the determination of the titer of the agent was desirable, since the conventional method, consisting of LDH assays on each of a set of 5 individual mice (5), was time-consuming. Therefore, a rapid method was devised in which LDH assays were performed on pooled plasma of 5 mice. It will be seen, that this method can be used when utmost precision is not required.

The scale representing the stepwise increase in LDH activity contributed by each additional infected mouse was determined as follows: Sixty-seven individual control mice were bled and the LDH activity in each plasma was determined. The mean concentration was 740 ± 29[1] LDH units/ml, the values ranging from 400 to 1400 units/ml.

Similarly, the mean concentration of 68 infected mice was 5128 ± 75[1] LDH units/ml, the values ranging from 3700 to 7000 units/ml.

From these data a table was constructed showing the expected LDH content of pools of plasma from groups of 5 mice with various numbers infected, and the corresponding standard deviations (Table I *a*). These values were used to form

[1] ± Standard error of the mean.

a system of classes which minimized the chance of a classification error and from which the most probable number of mice infected in each pool could be estimated. The ID_{50}/ml of the virus preparation was then calculated by the method of Karber (11). In Table I *b* are shown comparisons between the actual numbers estimated from the pooled plasma of the same mice, and the corresponding esti-

TABLE I *b*

Comparisons Between Actual Number of Mice Infected, and the Number Estimated from the Pooled Plasma of the Same Mice

	LDH Units/ml Plasma					ID_{50}/ml (Log 10)
Log dilution	−8	−9	−10	−11	−12	
Experiment 1						
Mouse 1	5000	4800	400	600	900	
2	4500	4000	400	500	500	
3	5400	4000	1300	500	600	
4	6000	5200	4900	900	600	
5	5200	4700	—	500	800	
No. infected	5	5	1	0	0	9.75 ± 0.25*
Pooled plasma	4600	4500	1600	600	600	
Estimated No. infected	4	4	1	0	0	9.35 ± 0.38
Experiment 2						
Mouse 1	6000	5200	1000	900	600	
2	4300	1000	1000	5400	500	
3	4600	4400	4800	500	700	
4	4600	5500	5400	6000	700	
5	5700	4800	4000	5800	900	
No. infected	5	4	3	3	0	10.5 ± 0.40
Pooled plasma	4500	3500	2800	3700	500	
Estimated No. infected	4	3	2	3	0	9.9 ± 0.47
Experiment 3						
Mouse 1	5100	5100	600	600	900	
2	4300	5000	6300	1000	700	
3	4600	5600	900	800	700	
4	4900	6000	1200	600	1000	
5	4200	5300	8000	600	600	
No. infected	5	5	2	0	0	9.9 ± 0.25
Pooled plasma	4400	5200	2900	600	600	
Estimated No. infected	4	5	2	0	0	9.7 ± 0.32

* Standard deviation.

TABLE I *c*

Comparison between actual number of mice infected, and the number, estimated from the pooled plasma of different mice, inoculated with the same virus dilutions (experiments 1 and 2), and a comparison of the number infected, estimated from the pooled plasma of two sets of mice inoculated with duplicates of the same virus dilutions (experiments 3 and 4).

	LDH Units/ml Plasma							ID_{50}/ml (Log 10)
Log dilution	−6	−7	−8	−9	−10	−11	−12	
Experiment 1								
Mouse 1	4600	6000	4500	5000	1000	—	—	
2	4800	6200	1300	6000	1000	—	—	
3	5400	4600	4500	5000	7000	—	—	
4	5000	5900	5300	5000	1100	—	—	
5	4600	5800	5100	5000	5100	—	—	
No. infected	5	5	4	5	2	—	—	9.7 ± 0.32*
Pooled plasma	6200	5700	4600	5100	1900	—	—	
Estimated No. infected	5	5	4	5	1	—	—	9.5 ± 0.28
Experiment 2								
Mouse 1	—	—	4500	4500	500	900	700	
2	—	—	4700	5300	4500	600	700	
3	—	—	3700	4200	4400	1200	800	
4	—	—	4300	4500	900	900	600	
5	—	—	5500	4600	5500	600	500	
No. infected	—	—	5	5	3	0	0	10.1 ± 0.25
Pooled plasma	—	—	5000	6500	4000	1100	600	
Estimated No. infected	—	—	5	5	4	1	0	10.5 ± 0.28
Experiment 3								
Pooled plasma	—	—	5900	4500	900	900	900	
Estimated No. infected	—	—	5	4	0	0	0	9.3 ± 0.20
Pooled plasma	—	—	6000	6800	2100	1000	900	
Estimated No. infected	—	—	5	5	2	0	0	9.9 ± 0.25
Experiment 4								
Pooled plasma	—	—	5200	4000	5300	800	1000	
Estimated No. infected	—	—	5	4	5	0	0	10.3 ± 0.20
Pooled plasma	—	—	5300	5300	3300	800	900	
Estimated No. infected	—	—	5	5	3	0	0	10.1 ± 0.25

* ± Standard deviation.

mates of the ID_{50}. In Table I *c* are shown similar comparisons made on parallel series of mice. In experiments 1 and 2 the two sets of mice were infected with the same virus dilutions, and the number of mice infected determined individually, or from pooled plasma. In experiments 3 and 4 two sets of mice were infected with duplicate dilutions of the same virus preparation and the number of mice infected determined from pooled plasma. It is seen that there is agreement to within about a half logarithm in the estimated ID_{50}'s, which is within the range of variability indicated by the standard deviations.

Growing the Agent in Quantity.—It was found possible to obtain relatively large quantities of the LDH agent by cultivating it in Ehrlich ascites tumor bearing mice.

CDF_1 mice were inoculated intraperitoneally with 0.05 ml of an LDH agent-free Ehrlich ascites cell suspension. After 7 days 0.1 ml of an LDH agent suspension ($ID_{50} = 10^{8.0}$/ml) was inoculated into the swollen peritoneal cavity. The ascitic fluid (5 to 12 ml/mouse) was collected 24 hours after inoculation with the agent, centrifuged, and the LDH agent titer of the resulting supernatant was found to be equal to that of the plasma (*i.e.*, $10^{9.5}$ to $10^{10.5}$/ml). Occasionally the stock Ehrlich ascites carcinoma became contaminated with the LDH agent, resulting in low titers as are found in permanently infected animals. Such strains could be freed of the agent by passing them once or twice through 7-day-old suckling rats, which maintain the Ehrlich carcinoma but not the LDH agent (An Ehrlich ascites line is routinely maintained in suckling rats and used when the mouse strain is found contaminated). This method of decontamination was previously reported by Rowson (12).

Partial Purification of the LDH Agent.—Early attempts to purify the LDH agent were unsuccessful. This was found to be due to the inactivation of the agent by the saline used for resuspension of sedimented material. However, at low temperature the LDH agent is stable in either complex solutions or distilled water (5). Subsequently, crude preparations were stored in Eagle's medium with 20 per cent veal broth, and the preparations obtained after hyaluronidase digestion (see Materials and Methods) in distilled water. The latter preparations have been used for the studies presented here.

As a first step to further purification, the effect of lipid solvents was studied. The inactivating effect of several organic solvents is presented in Table II. It can be seen that only after petroleum ether (PE) (petroleum benzin, Merck and Co., Inc., Rahway, New Jersey) extraction was the infectivity maintained. With petroleum ether (ligroine) a loss of one to two logs in infectivity was found. In all other solvents, a loss of 5 to 7 logs in infectivity occurred. Gas chromatography of the PE extracts from the LDH agent pellets indicated the presence of a number of free and esterified unidentified fatty acids of short chain length (C_{16} or less). The remaining pellet residue was devoid of fats extractable in a mixture of one volume methanol and 2 volumes chloroform. Ap-

parently the PE-extractable lipids are not essential for maintenance of infectivity. The degree of purification obtained by the hyaluronidase treatment, followed by PE extraction is indicated by the results, presented in Table III, in which the titers and the nitrogen contents of crude LDH agent, hyaluronidase-treated LDH agent, and PE-extracted LDH agent are given. Further purification of the PE-treated fractions is in progress.

Effect of RNase on crude and PE-extracted LDH Agent.—Since it has been reported that an ether extract or cold phenol extract of the agent was infective

TABLE II

*Effect of Organic Solvents on LDH Agent**

Solvent‡	Original titer ID_{50}/ml (Log 10)	Titer after treatment§ ID_{50}/ml (Log. 10)	Loss in infectivity (Log 10)
PE	9.5	8.7	0.8
PE	9.9	9.5	0.4
PE	9.5	9.3	0.2
PE	10.5	10.5	0.0
PE	10.5	10.1	0.4
PE	10.5	10.7	+0.2
PE	10.3	10.5	+0.2
EE	9.5	<3.0	6.5
EE	8.9	3.9	5.0
Alc.	8.9	2.5	6.4
Alc.	9.5	3.5	6.0
$CHCl_3$‖	9.5	<3.0	6.5
$CHCl_3$	8.9	2.1	6.8

* Hyaluronidase-treated preparations, resuspended in distilled water.

‡ PE, Petroleum ether (Petroleum benzin, Merck); EE, Ethyl ether; Alc., Ethyl alcohol.

§ All samples were shaken manually with 5 volumes of solvents for 5 minutes at room temperature.

‖ $CHCl_3$, +4 per cent isoamyl alcohol.

and that this infectivity was destroyed by RNase, and not by DNase (3, 13), similar experiments were performed on the PE-purified LDH agent preparation. The results (Table IV) show that RNase did not inactivate intact crude LDH agent, nor PE-extracted LDH agent, indicating that the PE had not removed a protective coat, although lipids were removed.

Morphology of the LDH Agent.—A number of hyaluronidase- and PE-purified pellets were prepared for electron microscopy. Figs. 1 *a* and 1 *b* show typical particles found in pellets of purified LDH agent. These particles have never been observed in uninfected control preparations. The particles consist of a very dense inner ring of about 25 mμ diameter and a less dense and sometimes indistinct outer ring extending to about 50 mμ. Although most of the particles appear

free, they are frequently seen within varying sized single-membraned vesicles, the smallest of which (Figs. 1 *b* and 1 *c*) could easily be construed as representing a "tail" in a negatively stained preparation (14). Infrequently large double-membraned bodies have been observed to be filled with characteristic particles (Figs. 2 *a* and 2 *b*). The origin and possible significance of these membraned structures is under further investigation.

TABLE III

*Partial Purification of LDH Agent**

Experiment No.	Crude preparations		10-fold Concentrated			
			Hyaluronidase preparation		PE extracted preparation	
	ID_{50}/ml (Log. 10)	mg N/ml	ID_{50}/ml (Log. 10)	mg N/ml	ID_{50}/ml (Log. 10)	mg N/ml
1	9.7	3.0	10.5	0.15	10.5	0.11
2	9.9	2.8	10.5	0.14	10.1	0.13
3	9.9	3.0	10.5	0.48	10.7	0.42
4	9.5	3.7	10.3	0.38	10.5	0.40

* All preparations were digested for 60 minutes at room temperature with hyaluronidase, followed by extraction with petroleum ether (See footnote, Table II).

TABLE IV

Effect of RNAse on Crude LDH Agent and PE-Extracted LDH Agent

Experiment No.	Crude preparations	PE extracted	RNase treated
1	8.3*	—	8.7‡
2	—	5.5	4.9§
3	—	6.3	6.5§

* Incubated for 24 hours at 37° C.

‡ Incubated for 24 hours at 37° C, 100 μg RNase/ml.

§ Incubated for 1 hour at room temperature, 100 μg RNase/ml.

A clear-cut difference between a hyaluronidase-treated preparation and one from which the lipids were removed by additional PE extraction was not apparent by electron microscopy (Figs. 2 *a* and 2 *b*). This has also been noted with lipid-extracted mitochondria (15).

Size of LDH Agent.—Subsequent to the discovery of the LDH agent, conflicting reports concerning its size have appeared. Riley (16) has reported a diameter of 2 mμ, whereas Notkins and Shochat (5) reported a diameter of 55 mμ, Rowson *et al.* (17) one of 45 mμ and later, Bladen and Notkins (14) one of 69 × 76 mμ. Since our electron micrographs indicate that the LDH agent has a

diameter of about 50 mμ, experiments were performed with PE-extracted LDH agent using millipore filters. The results are presented in Table V. They show that no infectious material passed through filters with a 10 mμ pore size, and that a small amount of infectivity passed through the 50 mμ filter in only 1 of 3 experiments. These results are in contrast to those of Riley (16) who reported that the LDH agent passed through millipore filters of 10 mμ pore size.

TABLE V

Filtration of LDH Agent

Experiment No.	Original titer ID_{50}/ml (Log. 10)	Titer of filtrate ID_{50}/ml (Log. 10)				
		10 mμ	50 mμ	100 mμ	220 mμ	450 mμ
1	6.5	<2.0	2.1	3.5	—	—
2	6.9	<1.0	<1.0	<1.0	4.7	4.7
3	7.6	<1.0	<1.0	2.9	3.9	—

TABLE VI

Interferon Production Following Infection with LDH Agent

Days after infection	Virus titer ID_{50}/ml (Log. 10)		Interferon titer/ml	
	Experiment 1*	Experiment 2‡	Experiment 1	Experiment 2
1	9.9	9.7	24	7
2	9.1	—	3	—
3	—	7.7	—	<3
8	—	7.5	—	<3
14	—	5.9	—	<3

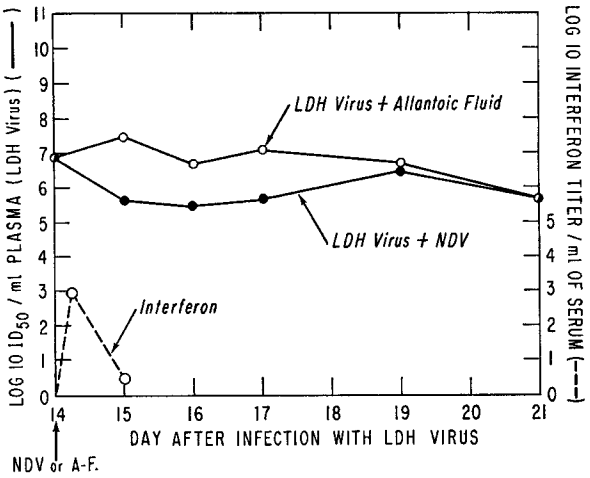
* Mice were inoculated i.p. with $10^{6.0}$ LDH agent.

‡ Mice were inoculated i.p. with $10^{8.5}$ LDH agent.

Besides the reports on variation in size of the LDH agent, Adams and Bowman (18) have reported that two different growth types of the agent exist, one reaching a maximum titer of 10^{11}, the other of 10^{7} ID_{50}/ml, which were separated by passing through a cellulose column, and by fractional sedimentation. In order to determine whether our preparations contained two strains, an LDH agent preparation not treated with hyaluronidase was centrifuged at 105,000 *g* for 2 hours. As Adams and Bowman found, 99.9 per cent was recovered from the pellet. However, in contrast to their findings, an additional 99.9 per cent of the agent remaining in the supernatant was recovered in the pellet after recentrifugation at 105,000 *g* for 18 hours. The final supernatant was injected into mice and found to give a typical growth curve with a titer of $10^{10.5}$ after 24 hours and of $10^{9.0}$ after 48 hours, indicating the absence of two types of LDH agents.

Antigenic Nature of the LDH Agent.—Since mice, after infection, remain permanently infected, as mentioned before, it might be expected that the LDH agent would be of low antigenicity. In order to study this problem, 2 rabbits and 2 guinea pigs were injected with the purified LDH agent ($10^{8.0}$ ID per ml).

For the first injection, the agent suspension was mixed with an equal amount of incomplete Freund's adjuvant. 0.25 ml of this mixture was injected in each foot-pad, and 1 ml intradermally. In addition, the rabbits received 1 ml intravenously, and the guinea pigs 1 ml intraperitoneally of the LDH agent suspension without adjuvant. Thereafter, the rabbits were injected intravenously and the guinea pigs intraperitoneally with 1 ml LDH agent suspensions at 7 and 14 days after the first injection and every 14 days thereafter for 14 doses.



TEXT-FIG. 1. Effect of NDV-induced interferon on the LDH agent titer in the plasma of mice. Time of injection of NDV or normal allantoic fluid indicated by arrow. NDV-induced interferon production is indicated by dotted line. Normal allantoic fluid did not produce interferon.

The sera were collected at seven 28-day intervals. Mice were inoculated with 100 infectious doses of LDH agent, mixed with equal amounts of either normal serum or of the two post-immunization sera, undiluted or in a 1:4 dilution. All groups of mice were subsequently found to be infected.

Apparently the LDH agent does not stimulate formation of neutralizing antibodies in rabbits or guinea pigs. These findings are in accordance with preliminary reports by others (19).

Interferon Production.—Since the LDH agent can be recovered indefinitely from mice once they are infected, it was of interest to determine whether the production of interferon by the LDH agent is low, thus allowing persistence of the agent. The interferon titer of mouse plasma was determined at various intervals after intraperitoneal infection with crude LDH agent. Table VI shows

that the interferon titer varied from less than 3 to 24 units. The technics of interferon assay and the relation of interferon production to LDH agent growth have been discussed elsewhere (20).

Further experiments showed that the LDH agent titer in the plasma of mice with an infection established for 14 days could be temporarily decreased following stimulation of circulating interferon by Newcastle Disease Virus.

One hundred and thirty mice were inoculated intraperitoneally with $10^{7.0}$ ID_{50} of LDH agent. Fourteen days later the pooled plasma of 10 mice contained $10^{6.9}$ ID_{50}/ml of LDH agent.

At this time 60 mice were inoculated intravenously with $10^{8.3}$ PFU of Newcastle disease virus, and 60 mice with allantoic fluid. At intervals of 1, 2, 3, 5, and 7 days the pooled plasma of 10 mice from each group was assayed for LDH agent concentration.

The results of a representative experiment, presented in Text-fig. 1, show that a temporary decrease in LDH agent titer of about 2 logs occurred after 24 hours, and about 1 log after 48 and 72 hours.

DISCUSSION

The rapid method of infectivity titration reported here is based on the LDH-determination of pooled plasma of 5 mice. Using such plasma, the following criteria should be observed: (*a*) young, healthy, female mice should be used, unless the male mice of the strain used are known to be docile and thus not infect cage mates by biting, as reported by Notkins, *et al.* (21); (*b*) plasma should be obtained 4 days after inoculation, since this is the period of maximal enzyme elevation (5); (*c*) DPNH solutions should be prepared fresh (22); and (*d*) hemolysed plasma should be discarded.

The cultivation of the LDH agent in Ehrlich ascites tumor-bearing mice made it possible to obtain quantities of the agent sufficiently large to show by electron microscopy that the diameter of the great majority of the particles was about 50 mμ. The observation that these particles were frequently seen in single-membraned vesicles of varying size and occasionally in large double-membraned bodies could explain the observation by Bladen and Notkins (14) who found a number of tailed particles, when the preparations were negatively stained. They might well represent the agent within small single-membraned vesicles. The presence of LDH agent particles within single- or double-membraned vesicles could also explain the observation by Crispens (23) that the small fraction of infectious particles, found in the supernatant fluid of his material after centrifugation in phosphate-buffered saline, was not inactivated by 1 M $MgCl_2$, if the vesicles have a lowered sedimentation velocity, and the vesicle walls protect the enclosed virus against $MgCl_2$. The presence of these vesicles could similarly explain the results by Adams and Bowman (18), who reported that sedimentable particles assayed up to 10^{11} ID_{50}/ml 20 hours after infection, and decreased to 10^{8} ID_{50}/ml in 14 days, whereas the relatively unsedimentable particles re-

tained their initial titer of about 10^7 ID_{50}/ml. In this case it could again be assumed that the vesicles are less sedimentable than the free particles although they are *larger*.

The presence of varying sizes of vesicles also would explain results by Adams and Bowman obtained by chromatography on DEAE-cellulose columns, since the vesicles would elute differently from the free particles, and lead to the elution peaks reported by them.

Neither our filtration experiments, nor the electron micrographs indicate the presence of an agent of such small dimensions as those based on filtration experiments through graded pores (16). Likewise, the results of filtration experiments obtained by Notkins and Shochat (5), by Rowson *et al.* (17), and the electron micrographs presented by Bladen and Notkins (14) do not confirm this smaller size. Our experiments also indicate that we do not have two LDH agent strains with different infectivity end-points.

The elevation of both glycolytic and TCA-cycle enzymes, including lactic dehydrogenase in the mouse plasma (4), could be understood if the observed vesicles represent loci of multiplication containing the enzymes either necessary for or produced as a result of the synthesis of the agent. The disintegration of these vesicles might then lead to an increased level of these enzymes in the plasma, especially since the agent decreases clearance of enzymes from the plasma (24, 25).

The gradual decrease of LDH agent titer to a constant value in permanently infected mice may be due to a permanent, low or undetectable level of interferon production. This was made plausible by experiments in which an increase of circulating interferon, brought about by subsequent inoculation with Newcastle disease virus, temporarily reduced the titer of the LDH agent.

SUMMARY

The lactic dehydrogenase agent was obtained in quantities sufficient for purification studies by growing the virus in Ehrlich ascites tumor-bearing mice.

A rapid method of titration of the agent is described.

Subsequent to the standard procedure of concentration of virus by treatment with hyaluronidase and centrifugation, lipids were removed by extraction with PE, without major loss of infectivity.

Electron microscopic sections of purified preparations contained particles consisting of a dense inner ring of about 25 mμ and a less dense ring extending to about 50 mμ.

The particles occur frequently in single-membraned vesicles of varying size, and occasionally in large double-membraned bodies.

The purified LDH agent did not stimulate the formation of neutralizing antibodies in rabbits and guinea pigs.

The crude LDH agent was found to be a low interferon producer.

Increased interferon, produced by secondary inoculation with Newcastle disease virus temporarily decreased the titer of the LDH agent.

The results of others regarding the nature and the size of the LDH agent are interpreted in regard to the findings presented, and the role of interferon in permanently LDH agent infected mice is discussed.

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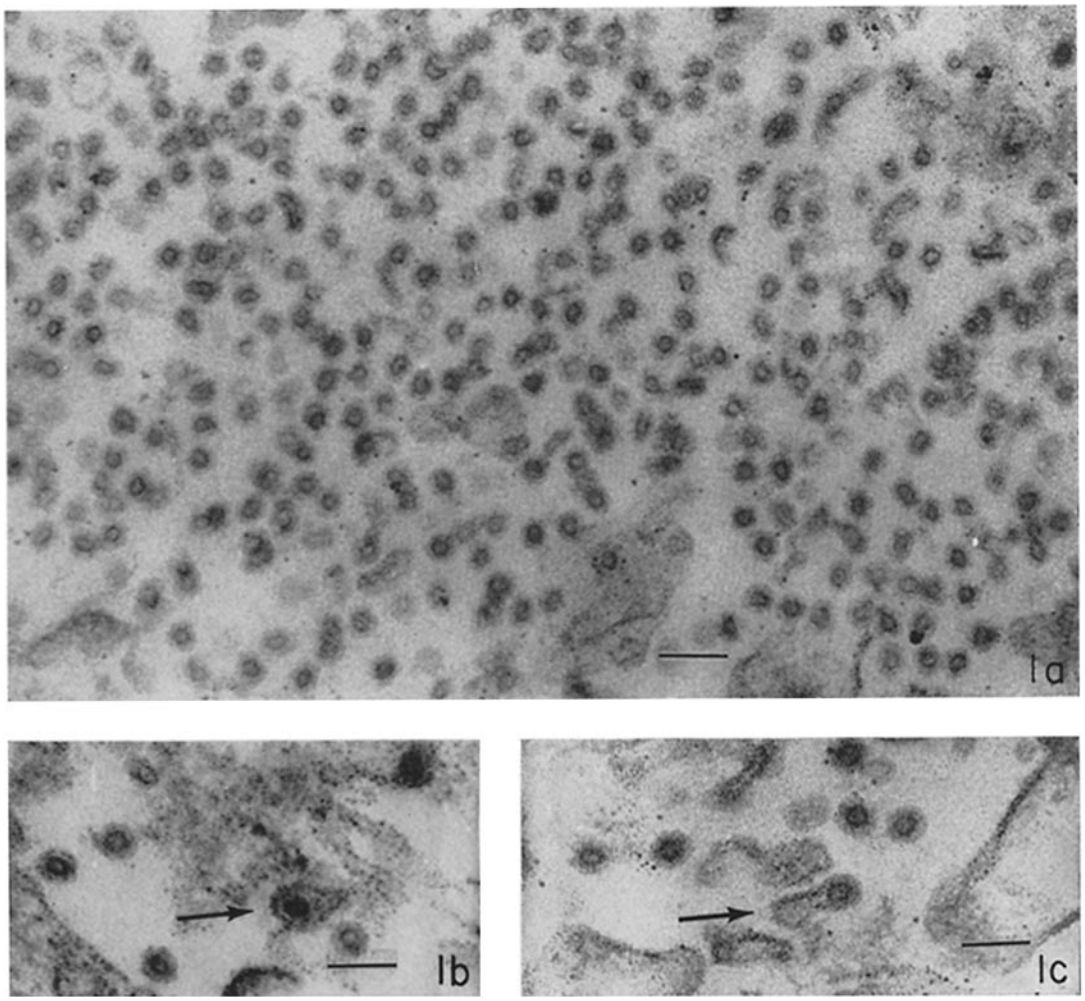
EXPLANATION OF PLATES

PLATE 37

FIGS. 1 *a* to 1 *c*. Sections of LDH agent pellets. (The lines represent 0.1 μ). × 98,700.

FIG. 1 *a*. Characteristic particles showing inner and outer rings.

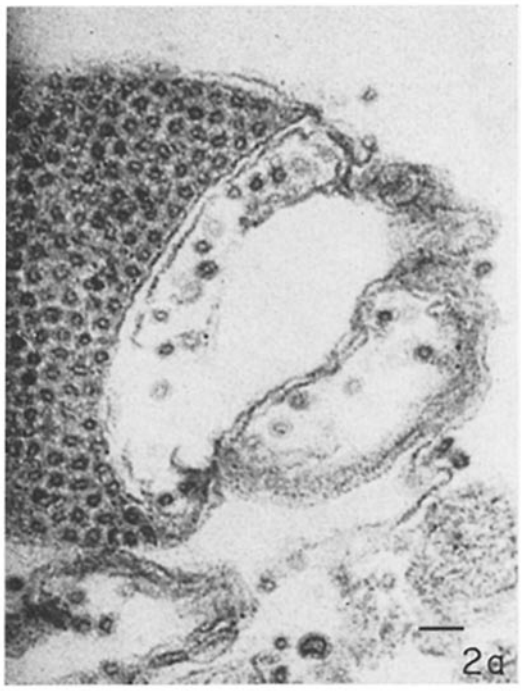
FIGS. 1 *b* and 1 *c*. Select areas each showing a single membrane-bounded particle.

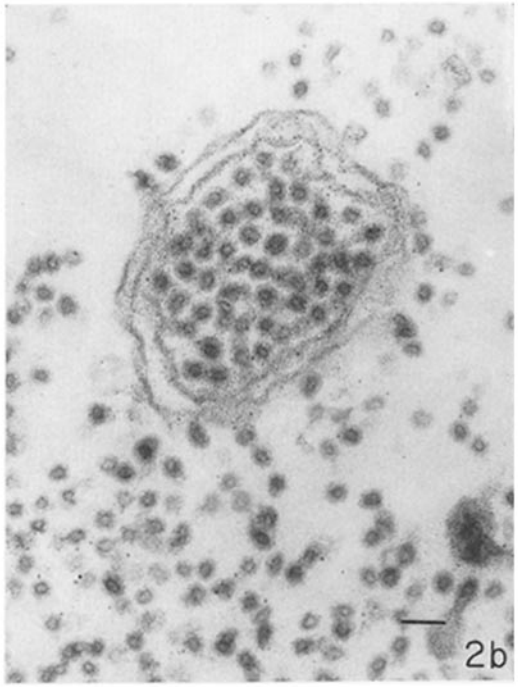


(du Buy and Johnson: Lactic dehydrogenase agent)

PLATE 38

FIGS. 2 *a* and 2 *b*. Ultra centrifuged pellets of hyaluronidase-treated LDH agent showing double membrane structures containing large numbers of characteristic particles. The preparation shown on the left (Fig. 2 *a*) was additionally treated with petroleum ether, that on the right (Fig. 2 *b*) was not so treated. (The lines represent 60 mμ). × 60,500.





(du Buy and Johnson: Lactic dehydrogenase agent)