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Plasma Lactic Dehydrogenase-Elevating Agent of Mice: Effect on Levels of Additional Enzymes.* (27911)

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Studies by Riley *et al.*(1) on changes of lactic dehydrogenase (LDH) in the plasma of tumor-bearing mice uncovered a virus-like agent which is associated with a high proportion of transplantable tumors in mice. The LDH-agent is recognized by its ability to initiate a 5-10-fold increase in the plasma LDH of infected mice. The agent is filterable, nondialyzable and thermolabile(1). It is readily transmissible in mice and persists in the plasma indefinitely without causing overt disease(2). The term virus is used in this communication in an operational sense since information on the true nature of the LDH-agent is lacking. The relationship of the LDH-agent with most transplantable mouse tumors appears to be that of a common contaminant(3-7). The foregoing observations indicated the importance of ascertaining whether the LDH-agent is responsible for alterations in the levels of additional enzymes in the plasma of infected animals and in relating such changes to the effect of tumor development in the absence of virus.

Materials and methods. Female mice of strain CFW, a partially inbred line of Swiss Webster mice, were used. Animals 4-5 weeks of age were received at weekly intervals from a commercial source† to minimize transmission of the agent in the local animal quarters.

Sarcoma 180 (S180) cells freed from the LDH-agent by serial propagation *in vitro* were used(7). The LDH-agent used in this study was isolated originally from a mouse bearing a S180 and carried through 12 passages in primary mouse spleen cultures(7). Concentrations of virus were determined by bioassay(8).

Samples of plasma were obtained by decapitating 4-7 mice and collecting the blood in a tube containing 0.3 ml of heparin solution (100 units/ml). Tubes were placed in an ice bath until the erythrocytes were removed by centrifugation at 1200 $\times$ *g* for 10 minutes at 4°C. The plasma was stored at −17°C until tested. Plasma showing evidence of hemolysis was discarded.

When mouse tissues were assayed for enzyme activities the mice were killed by decapitation. Tissues were removed immediately, weighed and homogenized in 20 volumes (v/w) of cold 0.067 M phosphate buffer, pH 7.4 in a teflon pestle glass homogenizer. Homogenates were centrifuged at 1000 $\times$ *g* for 5 minutes and the supernatants were employed for enzymatic tests.

Various enzymes were assayed as follows. The activities of lactic dehydrogenase, malic dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G-6-PD), alpha-glycerophosphate dehydrogenase (GPD, isocitric dehydrogenase (ICD), and glutathione reductase (GR) were determined by measuring the rate of change of optical density at 340 mμ resulting from oxidation or reduction of the appropriate pyridine nucleotide coenzyme(9). The detailed conditions for individual enzyme assays are presented in Table I. The measurements were performed with a Zeiss M4 QII monochrometer attached to an automatic Gilford cuvette

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† Carworth Farms, New City, N. Y.

TABLE I. Conditions of Assay for Various Pyridine Nucleotide-Dependent Enzymes.

		Components*							
Enzyme†	Plasma, ml	Coenzyme†	μM	Buffer, pH 7.5	μM	Cation	μM	Substrate	μM
LDH	.025	NADH	.35	Phosphate	175			Pyruvate	2.3
MDH	.025	NADH	.35	"	175			Oxalacetate‡	.75
GR	.1	NADPH	.35	"	175			Oxidized glutathione	3.2
GPD	.4	NADH	.35	Triethanolamine	100			Dihydroxyacetone-phosphate	.4
ICD§	.5	NADP	.80	Phosphate	135	Mn^{++}	5	DL-isocitrate	5.0
G-6-PD	.5	NADP	.80	Tris	100	Mg^{++}	10	Glucose-6-phosphate	4.0

* Total reaction mixture was 2.7 ml. All tests were conducted at 30°C. One unit of enzyme activity is defined as amount required to produce a change in optical density of 0.001 unit per minute.

† LDH = lactic dehydrogenase; MDH = malic dehydrogenase; GR = glutathione reductase; GPD = alpha-glycerophosphate dehydrogenase; ICD = isocitric dehydrogenase; G-6-PD = glucose-6-phosphate dehydrogenase; NAD = nicotinamide-adenine dinucleotide; NADH = reduced nicotinamide-adenine dinucleotide; NADP = nicotinamide-adenine dinucleotide phosphate; NADPH = reduced nicotinamide-adenine dinucleotide phosphate.

‡ Oxalacetate was free of pyruvate as checked with purified rabbit muscle lactic dehydrogenase.

§ No activity with NAD.

changer and optical density converter and a Leeds Northrup Speedomax H recorder. Appropriate colorimetric methods were employed for glutamic-oxalacetic (GOT)(10) and glutamic-pyruvic transaminases (GPT) (10), acid and alkaline phosphatases(11), leucine amino peptidase (LAP)(12), aldolase(13), and phosphohexose isomerase (PHI)(14). The tests were conducted and the enzyme units expressed as described in technical bulletins 505 (GOT, GPT), 104 (phosphatases), 250 (LAP), and 750 (aldolase) of Sigma Chemical Co. Plasma LDH was determined on each sample as an indicator of absence or presence of virus infection in the test mice. This was considered essential because the agent is highly contagious(8). Uninfected mice with and without tumors were kept isolated from virus-infected mice and considered to be free of the agent when their plasma LDH did not exceed 1800 units per ml(8).

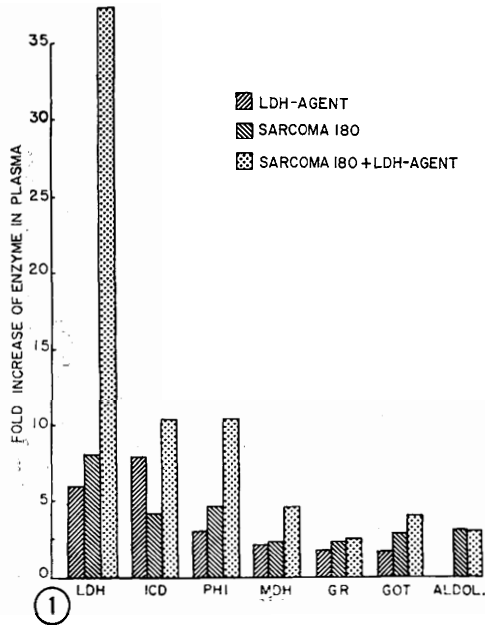
Results. The results presented in Table II indicate the activities of various enzymes in the plasma of normal and virus-infected mice, and of infected and normal mice bearing S180. Infection with the LDH-agent (column 2) results in a 5-10-fold increase of LDH and of ICD and in smaller elevations of PHI, MDH, GR, and GOT. The activities of aldolase, GPD, LAP, G-6-PD, GPT, and acid and alkaline phosphatases in plasma remained normal. During the final stage of development of S180 the following enzymes were elevated significantly in the plasma (column 3): LDH, PHI, ICD, aldolase, GOT, MDH, and GR. Alkaline phosphatase activity was decreased and GPD, G-6-PD, GPT, LAP, and acid phosphatase were unchanged. The absence of the LDH-agent in these mice was confirmed by bioassay of their plasma. It should be noted that both qualitative and quantitative differences in alterations of enzyme levels result from virus infection and from tumor development (Fig. 1). Elevations of most of these enzymes in the plasma of infected mice bearing the S180 (Table II, column 4) were approximately equal to the sum of the changes caused by either tumor or virus alone. A synergistic effect of virus plus tumor on the elevation of LDH and PHI, however, was observed (Fig. 1).

A study of the increase of enzyme activities in the plasma as a function of time following virus infection revealed that, except for PHI, all enzymes increased at a similar rate (Fig. 2). Enzyme levels in the plasma of infected mice remained elevated for at least one month. In an earlier study it was

observed that the LDH activity was undiminished 9 months post-infection(8). Distributions of GPT, LDH and ICD in various tissues of the mouse are presented in Table III. It should be noted that the activity of GPT is relatively high in the liver and low in the spleen and that erythrocytes contain only insignificant amounts of ICD. There was no difference in the activities of these enzymes in the tissues of virus-infected and uninfected mice.

Discussion. The results demonstrate that a number of enzymes in addition to LDH become elevated in mice as a result of infection with the LDH-agent (Table II). Alterations in PHI, MDH, GOT and GR are less pronounced than those observed with LDH and ICD. The activities of these enzymes were found to increase at about the same rate following infection (Fig. 2), suggesting that their release into the plasma is the result of a single process. As shown previously(8), maximum production of virus precedes by 1-2 days the elevation of enzyme activities in the plasma. The same enzymes that became elevated as a result of infection with the LDH-agent were found to increase in the plasma of tumor-bearing mice (Table II). Quantitative differences in the level of various enzymes, however, were observed between tumor bearing mice and those infected with virus (Fig. 1). Similar changes of GOT in the plasma of virus-infected and tumor-bearing mice were reported recently by Notkins and Greenfield(15). These findings together with the observation that the LDH-agent is associated with the majority of transplantable tumors in mice(1,3-7) indicate the necessity for cautious evaluation of earlier studies on the effect of tumors on the levels of plasma enzymes in this species.

All of the enzymes which are elevated as a result of infection with the LDH-virus or growth of S180 are probably of cytoplasmic origin(16). The plasma ICD is identical with the extramitochondrial ICD in its requirement for NADP and differs from a mitochondrial form which is specific for NAD (17). Malic dehydrogenase(18) and GOT (19) also exist in cytoplasmic and mitochondrial forms which differ in various chemical and physical characteristics. Both soluble and particle-bound forms of MDH and GOT



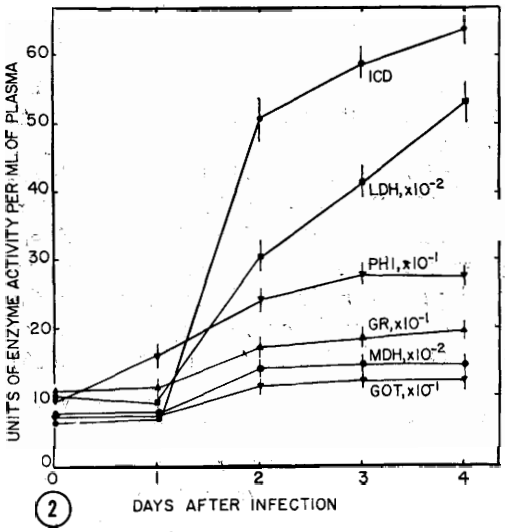


FIG. 1. Effect of infection with the lactic dehydrogenase-elevating agent and growth of sarcoma 180 on activities of enzymes in plasma of mice. Elevations of enzyme activities expressed relative to enzyme levels in normal mice.

FIG. 2. Increase in activities of enzymes in plasma of mice following infection with the LDH-elevating agent. One hundred mice were infected with 10^6 ID_{50} per mouse at zero time and 20 mice were sacrificed at daily intervals (4 mice per sample). Each value plotted represents the mean of 5 samples of plasma with its standard error. Twenty control mice sacrificed at end of experiment had normal levels of enzymes.

TABLE II. Effect of LDH-Agent and Sarcoma 180 on Levels of Plasma Enzymes in Recipient Mice.

Enzyme*	Units of enzyme activity per ml of plasma ± standard deviation			
	Normal	LDH-agent†	S180‡	S180 + LDH-agent§
LDH, x10^{-2}	7.7 ± 2.9 (100)‖	54 ± 13 (100)‖	83 ± 12 (33)‖	390 ± 70 (18)‖
ICD	18 ± 7 (44)	140 ± 28 (36)	72 ± 30 (9)	220 ± 60 (14)
MDH, x10^{-2}	8.1 ± 1.9 (41)	17 ± 4.6 (41)	20 ± 5.9 (14)	38 ± 15 (7)
GR, x10^{-1}	11 ± 2.2 (40)	18 ± 3.3 (40)	25 ± 6.1 (11)	26 ± 6.2 (11)
PHI, x10^{-1}	11 ± 2.0 (34)	34 ± 10 (33)	54 ± 13 (7)	120 ± 50 (13)
GOT	120 ± 31 (74)	180 ± 50 (94)	330 ± 150 (8)	460 ± 93 (13)
Aldolase	55 ± 13 (67)	60 ± 13 (66)	170 ± 16 (8)	164 ± 56 (21)
GPD	33 ± 17 (25)	49 ± 9 (25)	37 ± 15 (7)	46 ± 21 (21)
G-6-PD	31 ± 18 (35)	32 ± 19 (32)	28 (3)	40 ± 23 (14)
Alk. P.	2.9 ± .9 (11)	2.9 ± 1.0 (16)	1.3 ± .6 (16)	1.1 ± .3 (14)
Acid P.	.6 ± .2 (15)	.7 ± .3 (21)	.5 ± .2 (12)	.6 (3)
GPT	37 ± 6 (15)	38 ± 7 (17)	51 (3)	28 (3)
LAP	28 ± 9 (21)	32 ± 4 (24)	27 ± 6 (4)	25 ± 3 (10)

* The following abbreviations are employed: LDH = lactic dehydrogenase, ICD = isocitric dehydrogenase, MDH = malic dehydrogenase, GR = glutathione reductase, PHI = phosphohexose isomerase, GOT = glutamic-oxalacetic transaminase, GPD = alpha-glycerophosphate dehydrogenase, G-6-PD = glucose-6-phosphate dehydrogenase, Alk. P. = alkaline phosphatase, Acid P. = acid phosphatase, GPT = glutamic-pyruvic transaminase, LAP = leucine amino peptidase.

† Mice were injected intraperitoneally with $10^6 ID_{50}$ of LDH-agent/mouse and plasma enzyme levels determined 4-21 days post-infection.

‡ Mice were injected subcutaneously with 1-5 × 10^6 sarcoma 180 cells and plasma enzyme levels determined during final stage of tumor development (9-16 days after injection).

§ Mice were injected subcutaneously with 1-5 × 10^6 sarcoma 180 cells and intraperitoneally with $10^6 ID_{50}$ of LDH-agent/mouse. Plasma enzyme levels were determined during final stage of tumor development (9-16 days after injection).

‖ Values in parentheses indicate number of samples of plasma tested.

were found to be present in normal mammalian plasma, but excess levels associated with various disease states were contributed mainly by the cytoplasmic forms of both enzymes(19,20). The possibility that factors other than the intracellular location of enzymes are important in contributing to elevated plasma activities is indicated by the finding that such cytoplasmic enzymes as GPT, G-6-PD, GPD, LAP, and aldolase(16) remain relatively unchanged following virus infection. Tissue necrosis which is frequently cited as source of enzyme in plasma as a result of various diseases, probably does not contribute to alterations in activity observed in mice infected with the LDH-agent. Changes in permeability of either the cells in which the virus multiplies or of cells which may interact with it are probably responsible for the release of cytoplasmic enzymes into

TABLE III. Enzyme Activities of Mouse Tissues.

	Glutamic-pyruvic transaminase Units/mg wet wt*		Lactic dehydrogenase Units/mg wet wt*		Isocitric dehydrogenase Units/mg wet wt*	
	Normal	LDH-agent†	Normal	LDH-agent†	Normal	LDH-agent†
Liver	106 ; 64	90 ; 70	470 ; 380	380 ; 410	27 ; 31	31 ; 29
Kidney	3.4 ; 4.0	3.4 ; 2.5	460 ; 430	460 ; 350	35 ; 34	31 ; 34
Heart	2.5 ; 2.7	3.6 ; 3.8	350 ; 410	350 ; 340	23 ; 25	24 ; 32
Lung	2.5 ; 2.6	2.5 ; 2.5	130 ; 120	110 ; 120	7.4 ; 6.6	6.0 ; 5.6
Skeletal muscle	1.6 ; 2.0	1.8 ; 1.8	590 ; 560	620 ; 590	5.0 ; 3.8	4.8 ; 5.0
Spleen	.48; .48	.44; .44	140 ; 120	130 ; 130	2.8 ; 2.9	2.8 ; 2.8
Erythrocytes	.68; .68	.77; .78	73 ; 78	76 ; 70	.057; .050	.057; .060
Plasma‡	.037	.038	.77	5.4	.018	.140

* Each value represents the mean of duplicate determinations on a preparation from a single animal.

† Mice were injected intraperitoneally with 100 ID_{50}/mouse 5 days prior to test.

‡ Values of enzyme activity of plasma presented in Table II are included for comparison.

the plasma. Information on the potential effect of the LDH-agent on production of enzymes in cells is not available. A comparison of the LDH-isozyme patterns of plasma of normal and virus-infected mice showed that only the form which predominates in spleen, liver and erythrocytes was elevated(8). It is unlikely, however, that the erythrocytes are a major source of enzymes in the plasma since mouse erythrocytes do not contain significant amounts of ICD‡ (Table III). Liver contains relatively high concentrations of GPT(21) (Table III) and this enzyme becomes preferentially elevated in the plasma as a result of various types of liver damage (21). Release of enzymes from the liver following virus infection is therefore unlikely because the activity of GPT in plasma remains unaltered in infected mice.

The increased enzyme activities in plasma resulting from virus-infection or growth of S180 appear to be derived from different sources because additive or synergistic alterations were observed in virus-infected, tumor-bearing mice (Fig. 1). Elevations in tumor-bearing mice may be the result of release of enzymes from the tumor cells *per se*(22), but the possibility that tumor growth may cause release of enzymes from normal body cells cannot be disregarded(23). The specific patterns of enzyme elevations associated with various disease states probably reflect the concentration and intracellular localization of the enzymes in affected cells. It is possible, however, that virus infection or tumor growth imposes stresses on the regulatory cells or organs of the body and thereby affects the levels of plasma enzymes indirectly. The results of preliminary experiments indicate that multiplication of the LDH-agent in primary cultures of mouse spleen, lung and embryo does not result in increased production of LDH or in accelerated leakage of this enzyme into the medium.

The synergistic increase of plasma LDH in virus-infected, tumor-bearing mice(6,15, 24) remains unexplained. No such synergistic effects were observed with the other enzymes studied, with the exception of PHI. The results are consistent with previous findings(3-7) which indicate that the infection of mice with the LDH-agent has no direct relationship to tumor development *per se*, and that the association of the virus with numerous transplantable mouse tumors is coincidental.

Summary. Infection of mice with the lactic dehydrogenase-elevating agent resulted in a 5-10-fold increase of lactic and isocitric dehydrogenases and a 2-3-fold elevation of malic dehydrogenase, glutamic-oxalacetic transaminase, phosphohexose isomerase and glutathione reductase in their plasma. Significant increases in enzyme activities were observed 2 days after infection and achieved maximum levels within 3-4 days. Infection with this agent had no effect on the levels of aldolase, alpha-glycerophosphate and glucose-6-phosphate dehydrogenases, acid and alkaline phosphatases, glutamic-pyruvic transaminase, and leucine amino peptidase.

Elevations in enzymes of plasma in mice bearing sarcoma 180 differed both qualitatively and quantitatively from those resulting from infection with the agent. At the terminal stage of tumor development, lactic dehydrogenase was elevated 10-fold, isocitric and malic dehydrogenases, phosphohexose isomerase, aldolase, glutamic-oxalacetic transaminase, and glutathione reductase 2-5-fold. Alkaline phosphatase was decreased, whereas the activities of the other enzymes studied were normal. The level of plasma lactic dehydrogenase in tumor-bearing mice infected with the agent was 3-5 times higher than that expected from the results with either tumor or virus alone. Such synergistic effect of virus and tumor was not observed with the other enzymes.

‡ K. F. Gregory, personal communication.

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Genetic Differences in Growth Potential on Amino Acid Deficient Diets.* (27912)

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The chicken, unlike many mammalian species, has a well-defined requirement for arginine(1). It has been observed over a period of many years that body-weight gains of growing chickens given rations containing insufficient amounts of arginine show great variation(2,3,4). This variation is so striking that, on diets containing about half the arginine level considered necessary for optimum growth of the average chick, it is not uncommon to find some birds growing at a near normal rate, while others only maintain themselves. Whether the ability to grow on low-arginine intake by some but not all birds is genetically conditioned within a randomly bred flock is thus a question.

McDonald(5) and Miller *et al.*(6) have shown a breed difference in conversion of methionine into cystine in chickens, and Hegsted *et al.*(7) found that White Leghorns had a higher arginine requirement than Barred Plymouth Rocks. Goodman(8) had reported strain differences among mice in utilization of the optical isomers of certain amino acids. In the present study we are reporting on the apparent heritability of differences in the growth pattern on an arginine-deficient diet for offspring from different dams in a randomly bred flock of Leghorns. Subsequently we also extended these inquiries to another amino acid (lysine).

Methods. Fifteen hens and one cockerel were taken at random from a flock of Cornell Randombred(9) Leghorn chickens. The hens were trapnested to identify the origin of each egg, and the eggs were incubated at regular intervals. All chicks that hatched were grown in battery brooders for 4 weeks on a casein-glucose ration that provided approximately half the quantity of arginine which is considered necessary for optimum growth of the average chick receiving a casein diet. At the end of the 4-week period all birds were sexed.

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