Immunol., 1942, v44, 101.
5. Franklin, E. C., Progress in Allergy, 1964, v8, 58.
6. Onoue, K., Yagi, Y., Pressman, D., Immunochemistry, to be published.
7. Ingraham, J. S., J. Infect. Dis., 1952, v91, 268.
8. Cohen, E., Neter, E., Mink, I., Norcross, B. M., Arch. Inter-Am. Rheum., 1959, v1, 75.
9. Cohen, E., Nisonoff, A., Hermes, P., Norcross, B. M., Lockie, L. M., Nature, London, 1961, v190, 552.
10. Taliaferro, W. H., Taliaferro, L. G., J. Infect. Dis., 1950, v37, 37.
11. Stavitsky, A. B., J. Immunol., 1954, v72, 360.
12. Tabachnik, M., Sobotka, H., J. Biol. Chem., 1960, v235, 1051.
13. Greenbury, C. L., Moore, D. H., Nunn, L. A. C., Immunol., 1963, v6, 321.
14. ———, Nature, 1964, v203, 1147.
14a. ———, Immunol., 1965, v8, 420.
15. Chan, P. C. Y., Deutsch, H. F., J. Immunol., 1961, v85, 37.
16. Jacot-Guillarmod, H., Isliker, H., Vox Sang., 1962, v7, 675.
17. Josephson, A. S., Franklin, E. C., Ovary, Z., J. Clin. Invest., 1962, v41, 588.
18. Benedict, A. A., Larson, C., Nik-Khah, H., Science, 1963, v139, 1302.
19. Shulman, S., Hubber, L., Witebsky, E., ibid., 1964, v145, 815.

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Plasma Enzyme Elevations with LDH Viruses from Different Tumors.* (30532)

J. MARTYN BAILEY AND DENNIS A. WIGHT
Department of Biochemistry, George Washington University School of Medicine, Washington, D.C.

Many transplanted mouse tumors have been shown to be contaminated with a virus-like agent which produces 5-10-fold elevations in plasma LDH within 3-4 days of infection (1-8). The virus may be transmitted in the absence of tumor by injection of infected plasma alone and virus titer reaches 10^{10}-10^{12} infectious units per ml of plasma within 24 hours. Increase in plasma LDH enzyme does not begin until approximately 30 hours after infection, reaching a maximum in about 4-7 days, at which time virus titer has decreased to about 10^{6}-10^{7}(4). Infectious virus and elevation in plasma LDH enzyme persist throughout the otherwise normal lifespan of the mouse. There is no overt tissue damage and level of LDH in the tissues remains normal(6).

Following initial confirmation of the increase in LDH, it was shown that certain other plasma enzymes were also elevated(2,9, 10). A comparison of the results obtained by 4 groups of workers, each using a different tumor as a source of virus(10,9,2,7), shows that although there is qualitative agreement in the pattern of enzyme elevations, there is considerable quantitative disagreement for the different viruses. This is particularly evident for phosphohexose isomerase, where elevations ranging from 2.1-fold with the Moloney leukemia derived virus(2) to 6.7-fold with the Sarcoma 37 agent(9) have been reported.

Recent work has shown that plasma clearance of certain injected enzymes is impaired in infected mice(11,12,13,14) and blockage of plasma clearance mechanisms has therefore been proposed as a general mechanism for the action of LDH viruses. If such a common mechanism underlies the action of the virus, identical enzyme elevations should be produced by viruses from different tumors. If the viruses differ in their action, viruses from different tumors may produce different enzyme elevations. The present paper describes experiments in which LDH viruses were isolated from 8 different mouse tumors. Relative plasma enzyme elevations in the same strain of mice were measured at intervals following infection with each of the 8 viruses.

Materials and methods. Chemicals were from Sigma Chemical Co. or Nutritional Biochemicals, Inc. Mice used for measurement

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TABLE I. Contamination of Transplanted Mouse Tumors with Infectious LDH Agent.

Tumor	Type	Plasma LDH units/ml: Tumor bearing host	Test mice injected with plasma: 0 time	1 week	Agent present
C3HA2	Spontaneous mammary tumor	1386	198	220	—
DBA/59	Mammary sarcoma	2993	213	1510	+
H1295	Hepatoma (spontaneous)	—	290	1000	+
SS	Solid ascites (virus derived)	552	270	260	—
SSA	Ascites form of SS	1700	210	255	—
CD1	Lymphoma	210	—	—	—
CD5	" 1961	4735	180	1200	+
CD5	" 1964	347	220	190	—
DB12	Mammary sarcoma	528	—	—	—
H134	Hepatoma (spontaneous)	—	295	340	—
C4461	Methyl cholanthrene induced lung carcinoma	—	350	1470	+
HE10297	Sarcoma (spontaneous)	—	320	450	—
EA	Ehrlich ascites carcinoma	3800	250	1210	+
ML	Moloney leukemia	—	310	1300	+
G. 8755	Squamous cell carcinoma	—	199	1246	+
5	1st generation spontaneous tumors	150-400	—	—	—
EA(VF)	Ehrlich ascites carcinoma virus-free from tissue culture	280	190	210	—
EA1	Spontaneous infection of EA(VF)	1700	229	1482	+
EA2	Spontaneous infection of EA(VF)	3500	206	1297	+

Mice carrying tumors which were either grossly palpable or in the terminal stages of growth were bled from the retro-orbital plexus. Plasma LDH was measured only on those samples having no visible hemolysis. Test mice were bled at zero time and again 7 days following injection of 0.01 ml of plasma from the tumor-bearing animals. Elevation in plasma LDH of test mice indicated presence of virus in the tumor-bearing host. This was confirmed by at least one more passage of the virus using plasma from an infected test mouse. Note: 1) Elevation in plasma LDH of tumor-bearing mice is usually but not always associated with presence of LDH virus (as for example with C3HA2 and SSA tumors); 2) Elevated LDH in plasma of EA Ehrlich ascites tumor, but normal levels of plasma LDH in the EA(VF) strain of this tumor recovered virus-free after passage in tissue culture(15); 3) Apparent loss of infectious agent from CD5 lymphoma between 1961 and 1964; 4) Apparent synergism in raising plasma LDH level when both virus and tumor are present.

of plasma enzyme elevations were Swiss Webster white females (Dierolf Farms) 6-8 weeks old. Mice bearing the transplanted tumors described in Table I, were kindly supplied by Doctors Morris Barrat, R. C. Greenfield and Howard Andervont, Nat. Cancer Inst. The Moloney leukemia-associated LDH virus was from Dr. A. L. Notkins, Nat. Inst. of Dental Research, and the Ehrlich ascites carcinoma from Dr. Frank Henderson (Univ. of Alberta). One virus was of unknown origin and was isolated from an infected Swiss Webster mouse found in stock laboratory animals. Another virus (EA1) was isolated from "virus-free" Ehrlich ascites tumor (EAVF) following 5 generations of transfer in the uninfected state, as described previously(15).

Measurement of plasma enzymes. Mice were bled from the retro-orbital cavity using small heparinized capillary tubes, as described by Riley(16). Plasma LDH was measured by the modification of the method of Wacker *et al*(17), described previously(6). Isocitrate dehydrogenase was measured by a modification of the method of Wolfson *et al*(18). The optimum Mn^{++} concentration for the TPN-linked cytoplasmic mouse enzyme was found to be 6 μM. The final assay system contained sodium DL isocitrate 20 μM; TPN 0.4 μM, $MnCl_2$ 6 μM and 0.05 ml plasma with 0.5 ml of Tris buffer pH 7.5 in a total volume of 1.75 ml. Phosphohexose isomerase was measured by the method of Bodansky (19).

Detection and isolation of LDH viruses. Pairs of mice bearing the particular tumor were bled and the plasma was pooled. One portion was used for measurement of plasma LDH and a second portion (0.01 ml) to inject normal mice. A 4-fold or greater increase in plasma LDH of test mice after 4-7 days was indicative of the presence of an

LDH agent in the original tumor. The virus thus obtained was passed at least once more through uninfected mice and a pool of stock infected plasma was prepared and stored frozen at −50°.

Time-course of plasma enzyme elevations. Groups of 4 mice were bled at 0 time and each group was then infected with one of the 8 isolated viruses by intraperitoneal injection of 0.01 ml of infectious plasma. Determinations of plasma LDH, ICDH and PHI were made on duplicate samples of plasma taken at zero time and at 4, 7 and 21 days after infection.

Results and discussion. Plasma LDH and infectious virus in tumor-bearing mice. Levels of plasma LDH in the tumor-bearing mouse which were elevated more than 5 times normal were found in 7/12 of the transplanted mouse tumors tested (Table I). In 5 of these 7, the elevation in plasma LDH was transferable to a second mouse using plasma alone, thus confirming presence of infectious virus.† In addition, for 3 other tumors in which plasma LDH was not measured directly because of hemolysis, infectious virus was also demonstrated by passage to a second mouse (Table I). These results confirm that a substantial proportion of long-transplanted mouse tumors is contaminated with an LDH agent. When the agent is removed from the tumor by tissue culture passage(15), as for the Ehrlich ascites virus (EAVF, Table I), the plasma LDH of the tumor-bearing animal remains in the normal range. In some tumors, therefore, elevation in plasma LDH is almost entirely due to the virus. In certain other tumors, however, for example C3HA2, SS and SSA ascites tumors (Table I), elevation in plasma LDH in the tumor-bearing mouse is not accompanied by presence of an infectious LDH virus and is presumably produced by the tumor *per se*. The synergistic effect between virus and certain tumors (*e.g.*, Ehrlich ascites tumor) may be related to the mode of action of LDH virus in blocking clearance of plasma LDH enzyme. Release of additional LDH enzyme from the tumor in a virus-infected mouse in which LDH clearance is impaired will result in an apparent synergism, as noted by other workers(2,4,10).

Table I also contains one example of a tumor, CD5 lymphoma, which has apparently, in the interval between 1961 and 1964, lost a contaminating virus, and 2 instances (Ehrlich ascites carcinoma EA1 and EA2) in which the virus had been removed by tissue culture passage of the tumor, but in which, despite strict precautions to prevent accidental contamination(15), infectious virus has subsequently reappeared. The influence of LDH viruses on the growth of the virus-free Ehrlich ascites tumor has been described(15). No infectious virus was present in the 5 first-generation spontaneous tumors which were tested. This is in agreement with the observations of Notkins *et al*(3).

Plasma enzyme levels following infection. Plasma LDH, ICDH and PHI were all elevated at 4 days following infection, the average elevations being 5.1-, 6.1- and 3.6-fold, respectively. Essentially identical elevations resulted from infection with each of the 8 viruses (Table II). Levels of LDH and ICDH did not decrease significantly over the 4-21-day interval. This persistence in LDH elevation has previously been noted for periods of up to one year after infection(7). Plasma PHI values, however, reached a maximum at 4 days, then declined rapidly by the 7th day, returning close to normal levels by day 21 (Fig. 1). The range in values for PHI for the different viruses was considerably greater than for the other two enzymes (Table II). Small differences in the time-course of PHI elevation in the 0-7-day interval may in part be responsible for this wider range. This is also the most probable explanation for the variations in the previously reported elevations for this enzyme by other workers(10,9,2,7).

Probability that LDH agents operate by identical mechanisms. Both the pattern of elevation and the absolute magnitude of the elevation of the 3 enzymes tested are essen-

† *E. coccoides* has also been shown to cause elevated levels of plasma LDH due to lysis of RBC's. Infection with this agent, however, leads to a different time-course in plasma LDH elevation (22,23), and in addition does not give significant ICDH elevation because of the negligible amount of this enzyme present in RBC's(11).

TABLE II. Plasma Enzyme Levels Measured at Intervals Following Infection with LDH Agents from Different Tumors.

	Plasma enzyme levels (units/ml)											
	LDH				ICDH				PHI			
	Days following infection											
Virus type*	0	4	7	21	0	4	7	21	0	4	7	21
DBA-59	301	1354	1042	1199	28	269	255	246	170	550	505	234
EA	404	1216	1633	1363	25	282	349	243	108	474	443	170
EA1	263	1539	1547	1556	55	278	315	223	86	552	375	239
G 8755	199	1515	1246	1498	32	276	253	224	192	452	303	192
C 4461	300	1280	1846	1524	55	285	301	320	148	400	380	204
H 129-5	246	1356	1475	1487	39	304	229	259	78	476	341	183
ML	344	1446	1471	1631	66	296	287	246	216	672	564	200
Unknown origin	246	1304	1304	1407	32	303	302	231	96	335	382	179

* For description of tumor of origin see Table I.

Group of 4 Swiss Webster mice were infected by intraperitoneal injection of 0.01 ml of infectious plasma containing viruses from each of the 7 tumors listed, and also with one virus (unknown origin above) isolated from a contaminated stock mouse. Mice were bled from the retro-orbital plexus at 0 time and again at 4, 7 and 21 days following infection. Plasma samples were pooled and duplicate determinations of LDH, ICDH and PHI were made on each of the pooled samples.

tially identical following infection with each of the 8 viruses. The primary mode of action of certain of the LDH agents in elevating plasma enzymes has been shown to involve depression of host enzyme-clearance mechanisms. The results reported here therefore indicate that this mechanism is probably the same for all the LDH viruses. There is, however, some evidence that the agents themselves are not necessarily identical(20,21). Return of plasma PHI towards normal levels at 21 days may be related to return of the clearance of this enzyme to normal. It has been shown by Notkins(12) and Mahy(13) that RES function (as measured by clearance of colloidal carbon) is also depressed up to 75% in the first 24 hours following infection but rapidly returns to normal by about the 4th day. Impairment in plasma LDH clearance, however, apparently persists indefinitely.

Summary. 1. A number of transplantable mouse tumors were examined for presence of LDH virus by measurement of plasma LDH in the tumor-bearing host and by injection of plasma from the tumor-bearing animal into test mice. In the majority of cases a high plasma LDH in the tumor-bearing animal was associated with presence of the virus. 2. Plasma levels of LDH, ICDH and PHI were measured at 4, 7 and 21 days following infection with viruses from 8 different tumors. Essentially identical elevations of LDH (5.1-fold), ICDH (6.8-fold) and PHI (3.6-fold) resulted from infection with each of the 8 agents. 3. The results suggest a common mechanism of action of LDH viruses and are consistent with observations that the agents depress host plasma enzyme clearance mechanisms.

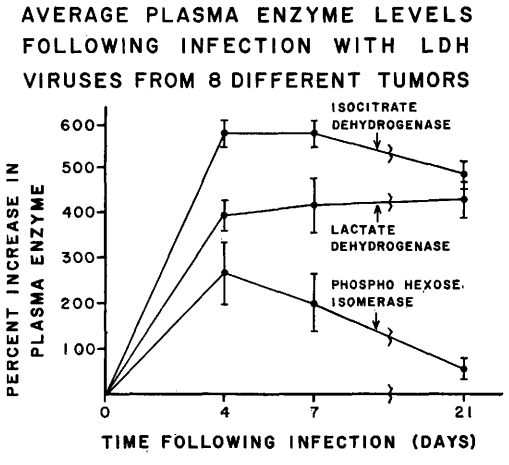

FIG. 1. Percentage increases in plasma enzyme levels calculated from data of Table II and expressed as increase over average normal plasma levels for each of the 8 viruses tested. Height of vertical line represents two standard deviations: Note 1) The rapid elevation in all 3 enzymes followed by the subsequent decrease in PHI over the 7-21-day period; 2) The maintenance of elevated ICDH and LDH over at least 21 days. In other experiments elevation in plasma LDH has been shown to persist for periods of at least 1 year.

1. Riley, Vernon, Lilly, F., Huerto, E., Bardell, D., Science, 1960, v132, 545.
2. Notkins, A. L., Greenfield, R. E., Marshall, D., Bane, L., J. Exp. Med., 1962, v117, 185.
3. Notkins, A. L., Greenfield, R. E., Berry, R. J., Moloney, J. B., Nature, 1962, v193, 79.
4. Riley, V., Ann. N. Y. Acad. Sci., 1963 v100, 762.
5. Plagemann, P. G. W., Swim, H. E., Proc. Am. Assn. Can. Res., 1963, v4, 53.
6. Bailey, J. M., Clough, J., Stearman, M., Proc. Soc. Exp. Biol. and Med., 1963, v114, 148.
7. ———, ibid., 1964, v115, 642.
8. Adams, D. H., Rowson, K. E. K., Salaman, M. H., Brit. J. Cancer, 1961, v15, 860.
9. Mahy, B. W. J., Rowson, K. E. K., Parr, C. W., Nature, 1963, v198, 885.
10. Plagemann, P. G. W., Watanabe, M., Swim, M. E., Proc. Soc. Exp. Biol. and Med., 1962, v111, 749.
11. Bailey, J. M., Clough, J., Stearman, M., ibid., 1964, v117, 350.
12. Notkins, A. L., Scheele, C., J. Nat. Cancer Inst., 1964, v33, 741.
13. Mahy, B. W. J., Virology, 1964, v24, 481.
14. Riley, V., Loveless, J. D., Fitzmaurice, M. A., Siler, W. M., Life Sciences, 1965, v4, 487.
15. Bailey, J. M., Clough, J., Lohaus, A., Proc. Soc. Exp. Biol. and Med., in press.
16. Riley, V., ibid., 1960, v104, 751.
17. Wacker, W. E. C., New Eng. J. Med., 1956, v255, 449.
18. Wolfson, S. K., Williams-Ashman, H. G., Proc. Soc. Exp. Biol. and Med., 1957, v96, 231.
19. Bodansky, O., Cancer, 1954, v7, 1191.
20. Cripens, C. G., Jr., Virology, 1964, v24, 501.
21. Bailey, J. M., Clough, J., Lohaus, A., Wight, D. A., Fed. Proc., 1965, v24, 597.
22. Riley, V., N. Y. State J. Med., 1963, 1525.
23. Notkins, A. L., Bact. Rev., 1965, v29, 143.

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Effect of Physiological Stress on the Delayed Hypersensitivity Reaction. (30533)

DONALD H. PITKIN (Introduced by John P. Ransom)
New England Institute for Medical Research, Ridgefield, Conn.

Delayed hypersensitive reactions can be produced in mice that have been sensitized by injection of water in oil emulsions of various proteins or by percutaneous application of minute amounts of certain chemicals (1-4,6). The severity of the reactions can be quantitatively assessed by measurement of the reacting zone and is decreased by pretreatment with the anti-inflammatory steroid cortisone(1,4,7). Passive transfer of serum from a sensitized donor to a normal recipient does not confer sensitivity to the recipient; however, transfer of immune lymphocytes can confer sensitivity. The factor responsible for sensitivity is considered a cell-bound antibody (1,5,6). The circulating level of cortisone or the reaction threshold to cortisone is altered in a stressed animal(7). These facts led to the hypothesis that stressed, sensitized animals would have a less severe delayed hypersensitivity reaction than would non-stressed sensitized animals. The results obtained in experiments designed to test this hypothesis are reported here.

Materials and methods. Male Swiss mice weighing 20-25 g at start of the experiment were used. They were fed mouse pellets and water *ad lib.*

The mice were sensitized by two 50 μg percutaneous applications of 1-chloro-2,4-dinitrobenzene* in freshly prepared acetone solution. The applications were made 14 days apart. The challenge dose, 20-25 μg, was applied 14 days after the second sensitizing dose.

Two types of stress were used: heating at 37°C for 60 minutes in a bacteriologic incubator or heating followed by intravenous injection of 0.2 ml of pyrogen free sterile saline.†

The reaction zones were measured with a millimeter rule to the nearest millimeter in 2 directions 90° apart; the resulting area was

* Distillation Products, Rochester, N. Y.
† Baxter Laboratories, Morton Grove, Ill.