12. R. W. Atchison, B. C. Casto, W. McD. Hammon, N. P. Rapoza, in preparation.
13. H. Hanafusa, T. Hanafusa, H. Rubin, *Proc. Nat. Acad. Sci. U.S.* **49**, 572 (1963); *Virology* **22**, 591 (1964).
14. M. D. Hoggan, *Federation Proc.* **24**, 248 (1965).
15. K. O. Smith and J. L. Melnick, *Science* **145**, 1190 (1964).
16. A. C. Allison, H. G. Pereira, C. P. Farthing, *Virology* **10**, 316 (1960).
17. S. Chandra and H. W. Toolan, *J. Nat. Cancer Inst.* **27**, 1405 (1961); H. W. Toolan, E. L. Saunders, E. L. Greene, D. P. A. Fabrizio, *Virology* **22**, 286 (1964).
18. J. P. O'Malley, *Proc. Exp. Biol. Med.* **108**, 200 (1961); personal communication, 1965.
19. A. J. Dalton, L. Kilham, R. F. Zeigel, *Virology* **20**, 391 (1963); C. H. Andrewes, in *Viruses of Vertebrates* (Williams and Wilkins, Baltimore, 1964), pp. 339–340.
20. T. B. Stim, F. C. Wissler, J. T. Grace, Jr., *Bacteriol. Proc.* **1964** 125 (1964).
21. Supported by PHS research grant No. CA-04574 from the NCI. We thank Barbara DiGregorio, Helen McCray, and Della Whitaker for technical assistance.

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Isoenzymic Specificity of Impaired Clearance in Mice Infected with Riley Virus

Abstract. *The clearance from the plasma of purified lactate dehydrogenase isoenzyme No. 5, which is increased in the plasma of mice infected with Riley virus, is impaired during the infection. That the clearance of purified isoenzyme No. 1 is unchanged by infection with Riley virus provides an explanation of why only the lactate dehydrogenase-5 isoenzyme activity is elevated in the plasma of infected mice.*

A viral infection of mice, characterized by a five- to tenfold increase in lactate dehydrogenase (LDH) plasma in the absence of other pathological changes, was first described by Riley *et al.* (*1*). The activities of a number of other enzymes, including isocitrate dehydrogenase, are also raised in the plasma of mice infected with Riley virus, whereas those of others, such as alkaline phosphatase and alanine transaminase, are not (*2*).

We have suggested that the increased plasma-enzyme activities of infected mice might be due to impaired clearance of endogenous enzymes from the plasma (*3*). Plasma clearance of intravenously injected LDH and isocitrate dehydrogenase is impaired in mice infected with Riley virus, whereas clearance of alkaline phosphatase and alanine transaminase is unaltered (*4*).

Studies on the patterns of LDH isoenzyme in the plasma of normal and of infected mice have shown that, of the five electrophoretically distinct forms normally present, only the slowest migrating (LDH-5) is increased in amount during Riley virus infection (*5*). The concentration of this isoenzyme is highest in liver, spleen, and skeletal muscle; the fastest-migrating LDH isoenzyme, LDH-1, on the other hand, is found in high concentration in heart tissue (*6*). It was therefore of considerable interest to determine whether the impaired clearance of intravenously injected LDH in mice infected with Riley virus was specific for the LDH-5 isoenzyme.

Male mice of a randomly bred albino strain, either uninfected or infected with Riley virus 4 to 6 days before, were used. The rate of clearance of injected LDH is depressed, and remains so from 3 to at least 20 days after infection (*4*). Mice were injected by way of the dorsal vein of the penis, and heparin was added to the blood samples which were obtained by bleeding from the tail. Plasma LDH clearance was determined by measuring the plasma LDH activities spectrophotometrically (*7*) at 2 minutes and at 3 hours after intravenous injection of purified crystalline LDH-1 (pig heart, Boehringer) or LDH-5 (rabbit muscle, Boehringer) (both at 1 mg/100 g of body weight) containing approximately 0.1 mg enzyme protein per milliliter of phosphate-buffered saline. As the specific activity of the LDH-5 preparation was about 2.5 times that of the LDH-1, a further experiment was carried out in which the LDH-5 isoenzyme (0.04 mg per milliliter of saline) was injected. This gave an initial LDH activity in the plasma similar to that produced by 0.1 mg of the LDH-1 isoenzyme per milliliter. The purity of the LDH-1 isoenzyme was stated to be more than 98 percent, and that of the LDH-5 isoenzyme to be more than 99 percent, relative to the other bands (*8*). The level of LDH in plasma immediately prior to LDH injection was also determined for each mouse, to give the activity at the "steady state." Clearance was then referred to the increase above the steady state at any time.

The clearance rate of LDH-1 was similar in the two groups of animals; thus there appears to be no inhibition of the clearance of this fast-moving isoenzyme in the infected mice. In the case of LDH-5, there was a highly significant inhibition of clearance in the infected animals. The uninfected mice cleared more than 60 percent of the injected LDH-5 isoenzyme, at either dose, within 3 hours, whereas the infected mice had cleared only 30 percent of the higher dose in this time, and none of the lower dose. Indeed, in the infected mice, the plasma LDH, instead of falling, had risen by 3 hours. This phenomenon of a rising LDH content, which we regularly observe in infected mice after small doses of LDH-5, must result from an increase in circulating endogenous enzyme. This could be due to an increased input resulting from some effect of the injected enzyme preparation such as cellular damage, or to a decreased clearance resulting from a blocking effect of the injected material. It might be expected that larger doses of injected enzyme preparation would produce a greater damaging or blocking effect than smaller doses. However, owing to the biphasic exponential nature of the rate of enzyme clearance (*4*), high doses of enzyme are cleared very much faster than small doses, and this high rate may mask small changes in endogenous enzyme input or clearance rate.

The rate of clearance of the LDH-1

Table 1. Clearance of plasma LDH-1 and LDH-5 (expressed as international units, IU) after intravenous injection into normal mice and mice infected with Riley virus.

Riley virus infection*	Plasma LDH (IU/ml × 10^{-3})			Clearance of injected LDH (%)	*P* between means (% clearance)
	Prior to injection	Excess over steady state† after			
		2 min	3 hr		
			LDH-1, 0.1 mg/100 g		
−	0.28 ± .03 ‡	2.10 ± .10	1.66 ± .10	21.0 ± 3.3	> .6
+	1.85 ± .09	1.85 ± .04	1.39 ± .14	25.1 ± 6.6	
			LDH-5, 0.04 mg/100 g		
−	0.39 ± .01	1.58 ± .13	0.57 ± .08	64.1 ± 5.0	< .001
+	2.00 ± .11	1.11 ± .06	1.40 ± .04	− 27.2 ± 4.5 §	
			LDH-5, 0.1 mg/100 g		
−	0.36 ± .03	5.60 ± .10	1.32 ± .17	76.2 ± 3.4	< .001
+	2.05 ± .06	6.19 ± .35	4.31 ± .13	29.6 ± 4.5	

* Mice were inoculated with Riley virus ($10^5 ID_{50}$), that is, 10^5 infective doses, 50 percent effective, 4 to 6 days before isoenzyme injection. † Obtained by subtracting the LDH activity of the steady state prior to injection from the observed activity at 2 minutes and at 3 hours. ‡ Values are means ± standard error of the mean of six observations in each case. § Percentage increase.

isoenzyme is much less than that of the LDH-5 isoenzyme in normal mice. This suggests that clearance of the two isoenzymes takes place by different mechanisms, but it may not be valid to compare clearance rates of isoenzymes derived from different animal species. However, Fleisher and Wakim have reported wide differences in the clearance rates of homologous glutamic-oxaloacetic transaminase isoenzymes in the dog (*9*).

Blockade of the reticuloendothelial system with thorotrast or cholesterol oleate causes increases in plasma enzyme of a pattern similar to those found in mice infected with Riley virus (*10*). Thus in blockaded, as in infected, mice LDH and phosphoglucose isomerase increased, whereas aldolase and alanine transaminase did not. Probably some plasma enzymes, or isoenzymes, are cleared by the reticuloendothelial system, whereas others are not.

Our results provide an explanation of why only the LDH-5 isoenzyme activity is elevated in the plasma of mice infected with Riley virus.

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References and Notes

1. V. Riley, F. Lilley, E. Huerto, D. Bardell, *Science* **132**, 545 (1960).
2. P. G. W. Plagemann, M. Watanabe, H. E. Swim, *Proc. Soc. Exp. Biol. Med.* **111**, 749 (1962); B. W. J. Mahy, C. W. Parr, K. E. K. Rowson, *Nature* **198**, 885 (1963); A. L. Notkins, R. E. Greenfield, D. Marshall, L. Bane, *J. Exp. Med.* **117**, 185 (1963).
3. B. W. J. Mahy, K. E. K. Rowson, M. H. Salaman, C. W. Parr, *Virology* **23**, 528 (1964).
4. B. W. J. Mahy, *ibid.* **24**, 481 (1964); J. M. Bailey, J. Clough, M. Stearman, *Proc. Soc. Exp. Biol. Med.* **117**, 350 (1964); A. L. Notkins and C. Scheele, *J. Nat. Cancer Inst.* **33**, 741 (1964).
5. P. G. W. Plagemann, K. F. Gregory, H. E. Swim, K. K. W. Chan, *Can. J. Microbiol.* **9**, 75 (1963); M. L. Warnock, *Proc. Soc. Exp. Biol. Med.* **115**, 448 (1964).
6. D. M. Dawson, T. L. Goodfriend, N. O. Kaplan, *Science* **143**, 929 (1964).
7. F. Wroblewski and J. S. La Due, *Proc. Soc. Exp. Biol. Med.* **90**, 210 (1955).
8. Starch-gel electrophoresis of the isoenzyme preparations showed that the LDH-1 isoenzyme, although contaminated with an appreciable amount of LDH-2, contained no detectable LDH-3, LDH-4, or LDH-5. The LDH-5 isoenzyme, although contaminated with an appreciable amount of LDH-4 and a visible trace of LDH-3, contained no detectable LDH-1 or LDH-2.
9. G. A. Fleisher and K. G. Wakim, *J. Lab. Clin. Med.* **61**, 98 (1963).
10. B. W. J. Mahy, K. E. K. Rowson, C. W. Parr, *Biochem. J.* **95**, 19 P (1965).
11. We thank Dr. C. W. Parr for carrying out starch-gel electrophoresis of the isoenzyme preparations, and Misses C. Reif and D. Read for valuable technical assistance. Partly supported by a block grant from the British Empire Cancer Campaign.

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Transformation by Rous Sarcoma Virus: A Requirement for DNA Synthesis

Abstract. *Transient inhibition of DNA synthesis immediately after infection of cells with Rous sarcoma virus prevents subsequent morphological transformation of the cells. Inhibition of DNA synthesis for an identical period before infection, or later in the course of infection, has only slight effect on transformation. Thus DNA synthesis is a specific requirement in the transformation of cells by Rous sarcoma virus.*

The conversion of a cell from a normal to malignant form must be accompanied by specific biochemical changes. The changes are deemed hereditary since tumor cells confer upon their progeny the continuing capacity for tumorigenesis.

One might suspect, therefore, that the conversion of a cell to the malignant form is dependent in some way upon DNA. The observation that actinomycin inhibits chemical carcinogenesis in animals (*1*), or conversion of cells by simian virus 40 (SV40) (*2*), has led these investigators to suggest that carcinogenesis requires DNA-dependent RNA synthesis, although a requirement for DNA synthesis was not excluded in the experiments.

Rous sarcoma virus (RSV) has a specific requirement for DNA synthesis early in its infectious cycle (*3–5*), although the virus presumably contains only RNA (*6*). Whether the requirement is for new cellular DNA synthesis or for a virus-primed DNA synthesis has not been resolved. This requirement for DNA synthesis is transitory since the growth of virus is unaffected if specific inhibitors of DNA synthesis are added later than 12 to 16 hours after infection (*5*). However, the completion of the virus as an infectious entity is unnecessary for transformation of chick-embryo cells by RSV (*7*). The following experiments demonstrate that DNA synthesis is necessary during the early stages of infection by RSV in order for cells to be transformed.

The methods for the growth of chick-embryo cells and for the propagation and assay of the Bryan strain of RSV have been described (*3, 8*). The test medium consisted of Eagle's medium No. 2 with 10 percent calf serum and antibiotics. Antiserum to RSV was induced in turkeys and prepared by University Laboratories, Highland Park, New Jersey. A dilution of 1 : 100 of this serum, as used in these experiments, was capable of inactivating more than 99 percent of a suspension of RSV containing 10^5 focus-forming units of RSV per milliliter during incubation for 30 minutes at 37°C.

Cells transformed by RSV are easily recognized if they occur in a mass culture or, more quantitatively, as a group (focus) against a background of normal cells, but they cannot be recognized with confidence if they occur singly. Cellular divisions are necessary for focus formation, and DNA synthesis is a continuous requirement for dividing chick-embryo cells. In order to test the effect of reduced DNA synthesis on transformation, it was necessary to use experimental conditions in which DNA synthesis resumed after a period of interrupted activity.

Cytosine arabinoside [1-β-D-arabinosylcytosine (ara-C)] effectively blocks

Table 1. Reversal of inhibition of DNA synthesis. Chick-embryo cell cultures were exposed to ara-C ($10^{-3.5}M$) for 4 hours at 37°C. The medium on some cultures was then replaced with medium containing deoxycytidine (dC, $10^{-3}M$). Cultures were exposed to H^3-thymidine (10 μc in 3 ml) for exactly 1 hour, and incorporation of H^3 into material precipitable by cold $HClO_4$ was measured (*5*).

Treatment	Addition of H^3 (hr after exposure to ara-C)	H^3 (count/min)
None	0	26,618
ara-C 5 hr	0	896
ara-C 4 hr, then dC	0	2,296
ara-C 4 hr, then dC	1	4,763
ara-C 4 hr, then dC	3	20,390
ara-C 4 hr, then dC	5	41,386

Table 2. Effect on virus yield of limited exposure of cells to ara-C. Cells were infected with RSV (about two focus-forming units per cell), washed, and exposed to ara-C ($10^{-3.5}M$) for 8 to 12 hours. Ara-C was removed, deoxycytidine ($10^{-3}M$) added, and culture fluids were removed at various later times.

Exposure to ara-C (hr)	Focus-forming units per culture at intervals after infection		
	24 hr	32 hr	36 hr
None	69,000	255,000	1,650,000
8	1,635	6,300	
12	<15		375