STUDIES ON THE MECHANISM OF ACTION OF RILEY VIRUS

IV. The Reticuloendothelial System and Impaired Plasma Enzyme Clearance in Infected Mice

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In mice infected with Riley virus (1), the activities of a number of plasma enzymes are permanently elevated (2, 3). Similar, although temporary, increases in plasma enzyme activity have been produced in mice by the administration of certain substances which reduce the phagocytic activity of the reticuloendothelial system (RES), and so it was suggested that in both cases the increased plasma enzyme levels might be caused by inhibition of plasma enzyme clearance by the RES (4). Previous studies in Riley virus–infected mice have demonstrated a reduced rate of plasma clearance for those enzymes which have an increased activity in the plasma of infected mice. The enzymes for which this relationship has been demonstrated are lactate dehydrogenase (LDH), isocitrate dehydrogenase, malate dehydrogenase, and aspartate transaminase (formerly called glutamic-oxalacetic transaminase) (5–7). With two other enzymes, alkaline phosphatase and the LDH-1 isoenzyme of LDH, the rate of clearance from the plasma was the same in normal and Riley virus–infected mice, and the plasma activities of these enzymes remain unchanged after Riley virus infection (6, 8).

The present study was undertaken to extend previous work on plasma enzyme clearance in Riley virus–infected mice, and to examine the effect of altered RES activity on plasma LDH clearance in noninfected mice.

Materials and Methods

Animals.—Randomly bred albino male mice of the Parkes strain were used at 6–8 wk of age.

Injections and Sampling.—Intravenous injections were made via the dorsal vein of the penis (9) and serial blood samples taken by tail bleeding (10). The technique of retro-orbital bleeding was found to be unsatisfactory for enzyme clearance studies, as repeated hourly sampling caused local tissue damage and resulted in misleading plasma enzyme levels.

Test Materials.—Crystalline LDH from rabbit muscle (C. F. Boehringer and Soehne, Mannheim, Germany), phosphoglucose isomerase from yeast (Boehringer and Soehne), and alanine transaminase (formerly called glutamic-pyruvic transaminase) from pig heart (Koch-Light

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Laboratories Ltd., Colnbrook, England) were centrifuged out of suspension and the deposit dissolved in normal saline. The enzyme concentration was adjusted so that the required dose of enzyme could be given in a volume of 1 ml per 100 g body weight intravenously to each mouse. Colloidal carbon, stilbestrol, and cholesterol oleate were prepared for injection as previously described (4).

Determinations.—Procedures for determining the activities of LDH, alanine transaminase, and phosphoglucose isomerase were as previously described (4). All enzyme activities are expressed as international units (11).

Riley Virus.—The virus preparation used in these experiments consisted of mouse plasma obtained from animals 24 hr after infection and diluted 1 in 100 in Hanks' saline containing 0.5% gelatin (4).

Enzyme Clearance.—The term clearance refers to the disappearance of enzyme activity from the peripheral circulation. Clearance rates were determined in individual mice by injecting purified enzyme intravenously and determining the plasma enzyme activity both prior to and at intervals up to 48 hr following injection. The initial plasma sample following injection was taken at 2 min, when mixing was assumed to be complete in the peripheral circulation. The enzyme activity in the plasma prior to injection was taken as the steady-state activity ($A\infty$) and the level 2 min after injection as the activity at zero time ($A0$). The clearance of enzyme activity from the plasma was generally found to follow an exponential curve, and could be described by the equation:

$$A = A0 \cdot e^{-Ct} + A\infty$$

where A is the enzyme level at any time t, and C is the clearance coefficient. Plots of $\log_e$ (A-A∞) against t should thus give a straight line, the slope of which is the clearance coefficient. In all cases, the best straight line was calculated by the method of least squares. However, in some experiments, the clearance curve was found to have two phases, and in these cases the best straight line was calculated for each phase separately.

RESULTS

Clearance of LDH in Normal and Infected Mice.—The clearance of LDH from the plasma was measured after intravenous injections of rabbit muscle LDH (50,000 IU/100 g body weight) in both normal mice and mice infected several days previously with Riley virus. The steady-state level (A∞) of plasma LDH was around 200 IU per liter in normal mice, and 8–10 times higher in the infected mice. A typical result is presented in Fig. 1.

In normal mice, the LDH clearance curve was biphasic and proceeded at a fast rate down to a level that varied between individual mice but was generally 800–1,000 IU per liter, then at a slower rate, to approach the steady-state level by 1–2 days. In Riley virus–infected mice, the initial fast clearance rate was either greatly reduced or did not appear at all (Fig. 1). The two clearance phases seen in normal mice must represent different processes. In the absence of more precise information as to their nature, these two removal mechanisms will be referred to as the "fast" and "slow" phases.

The fast clearance phase in normal mice may be the period during which the injected enzyme is coming to an equilibrium between the plasma and an extravascular compartment, such as the interstitial fluid or cells of the RES. The change from fast to slow clearance would occur when this equilibrium had

been established. If this were so, it should be possible to block the fast clearance phase, or alter the plasma enzyme level at which the change in rate occurs, by repeated injections of LDH. Fig. 2 shows the results of such an attempt. Two groups of uninfected mice were given intravenous injections of LDH,

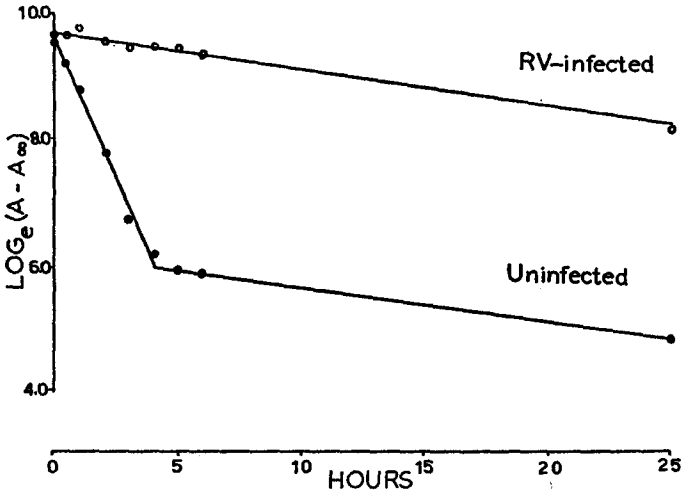

FIG. 1. Clearance of LDH from the plasma of an uninfected mouse, and a mouse which had been infected with Riley virus 10 days previously. Each mouse was injected with 18,000 IU/liter intravenously.

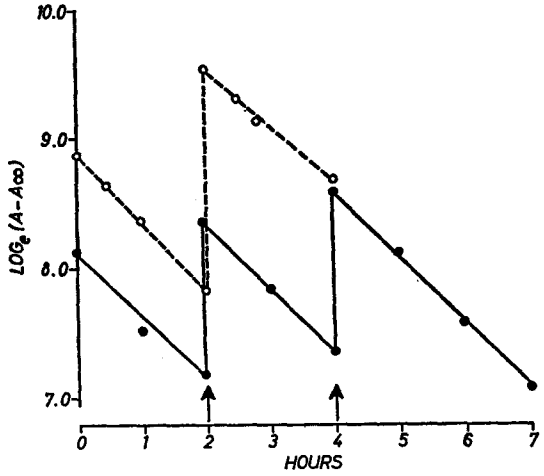

FIG. 2. Clearance of LDH from the plasma after intravenous injection of 3000 (●——●) or 7000 (O- - -O) IU/liter respectively into groups of normal mice. Each point represents a mean value obtained from four mice. Arrows show reinjection times. For further details see text.

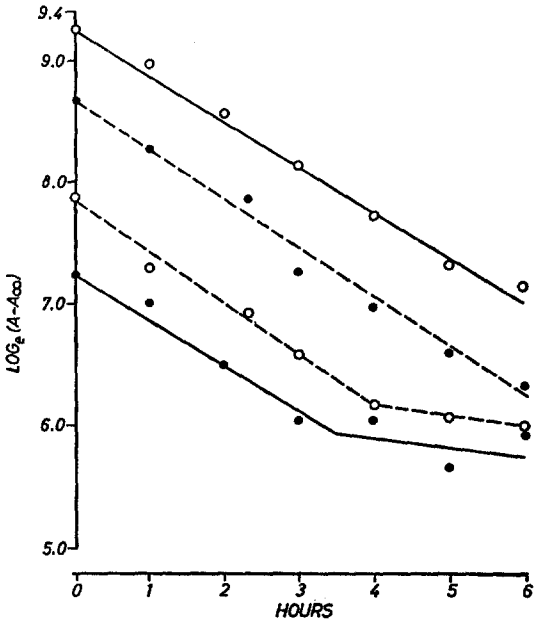

FIG. 3. Clearance of LDH from the plasma after intravenous injection of 1200 (●——●), 2500 (○- - -○), 6000 (●- - -●), or 10,000 (○——○) IU/liter respectively into groups of normal mice. Each point represents a mean value obtained from four mice.

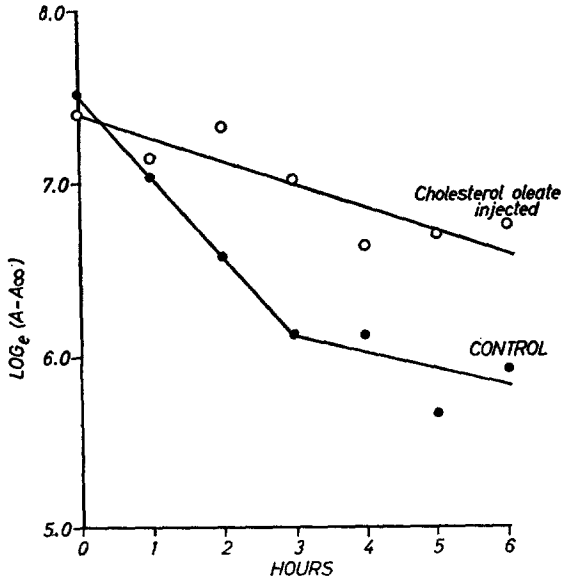

FIG. 4. Clearance of LDH from the plasma after injection of 1500 IU/liter intravenously into normal mice (●——●) and mice injected 24 hr previously with cholesterol oleate (○——○). Each point represents a mean value obtained from four mice.

each receiving a different dose. 2 hr later these doses were repeated, followed, in the case of the group receiving the lower LDH dose, by a third injection at 4 hr. All the clearance curves had the slope of the fast phase; thus there was apparently no saturation of the fast phase mechanism, nor was there any evidence that the change from fast to slow clearance phase was occurring at

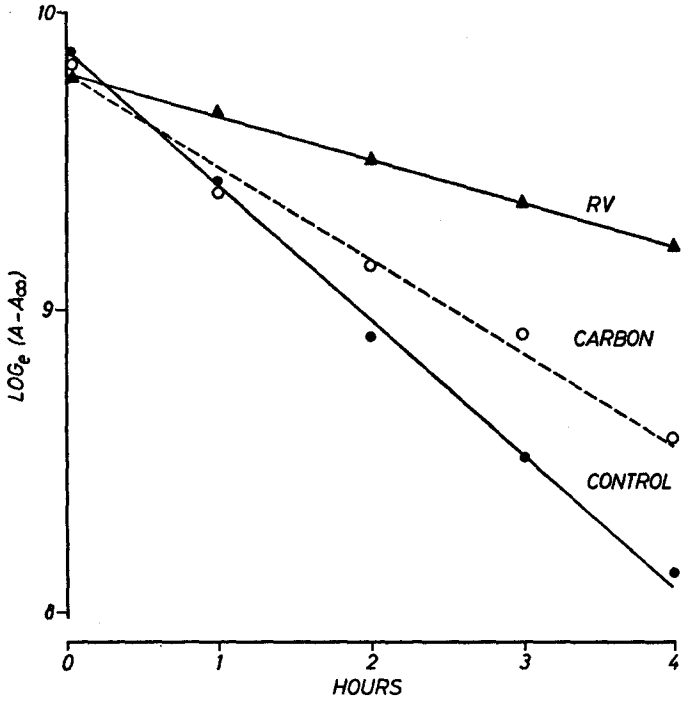

FIG. 5. Clearance of LDH from the plasma after injection of 18,000 IU/liter intravenously into a normal mouse (●——●), a mouse injected 1 hr previously with 16 mg/100 g body weight of carbon intravenously (O- - -O) and a mouse infected for several weeks with Riley virus (▲——▲).

an appreciably higher plasma enzyme level after the second or third enzyme injection than after the first.

Another possibility is that the switch from fast to slow clearance phase involves some type of threshold mechanism, so that enzyme is cleared at a fast rate only down to a critical plasma level. This hypothesis was supported by the following experiment. Groups of uninfected mice were given injections of LDH at various dose levels and the clearance studied for 6 hr (Fig. 3). The two higher doses were cleared at the fast rate throughout the observation period, at the end of which the plasma level was still above 800 IU per liter, where the switch from fast to slow clearance had been shown to occur. How-

ever, at the lower dose levels, two clearance phases were seen, and the switch from fast to slow clearance again occurred at a plasma level of about 800 IU per liter.

Relation of RES to Plasma LDH Clearance.—The injection of mice with RES-blocking agents causes an increase in the level of plasma LDH and certain other enzymes, which are the same enzymes as are affected by Riley virus infection (4). If the causative mechanism is the same in both cases, RES-

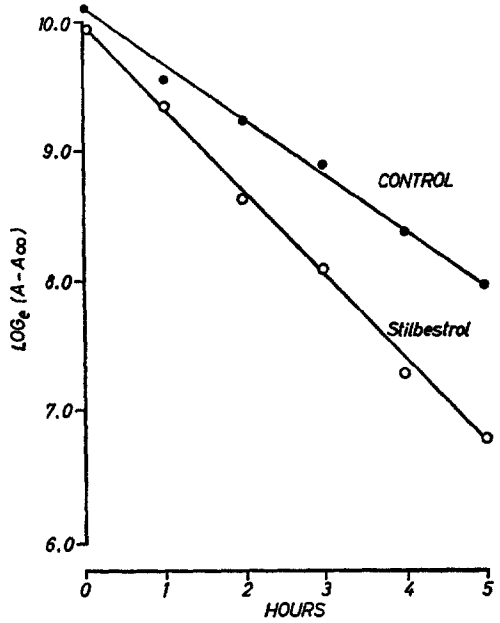

FIG. 6. Clearance of LDH from the plasma after injection of 21,000 IU/liter intravenously into groups of normal mice (●——●) and mice injected 2 days previously with stilbestrol (○——○). Each point represents a mean value obtained from three mice.

blocking agents should impair the fast LDH clearance phase in mice. To test this hypothesis, groups of mice were injected intravenously with either cholesterol oleate emulsion (100 mg/100 g body weight) or saline, and 24 hr later the clearance rate of LDH was measured following intravenous injection of enzyme (Fig. 4). The control mice showed the usual biphasic clearance curve, but in the cholesterol oleate–treated group, although the levels of plasma LDH were somewhat variable, only the slow clearance phase was seen.

A similar experiment was carried out in which the function of the RES was blocked by an intravenous injection of carbon (16 mg/100 g body weight) 1 hr prior to studying LDH clearance. At this time, RES function as measured

by carbon clearance was somewhat reduced, though not so greatly as after cholesterol oleate (4). The rate of LDH clearance was compared in three mice, one normal, one treated with carbon, and one infected with Riley virus several weeks previously (Fig. 5). The dose of LDH chosen was high, so in the normal mouse only the fast clearance phase was observable over the period studied. Carbon injection reduced the rate of LDH clearance but not to so great an extent as Riley virus infection.

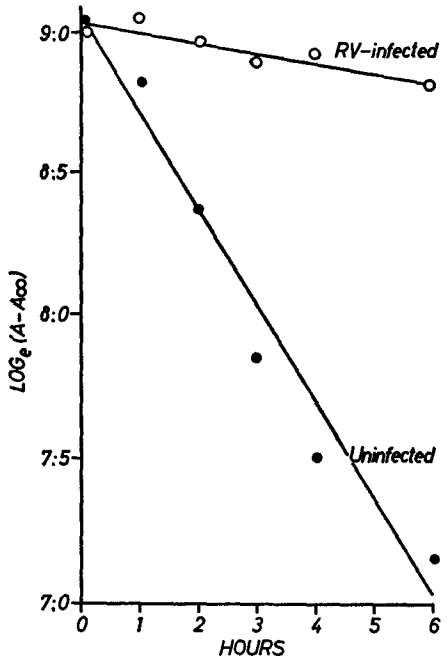

FIG. 7. Clearance of phosphoglucose isomerase from the plasma after intravenous injection of 8000 IU/liter into normal mice (●——●) and mice infected for several weeks with Riley virus (O——O). Each point represents a mean value obtained from four mice.

Stimulation of the RES with stilbestrol lowers the plasma LDH level in mice (4), and it was therefore of interest to determine the effect of this substance on LDH clearance. Mice were given a single subcutaneous injection of 0.1 mg stilbestrol in arachis oil, controls receiving arachis oil alone. This dose had been shown to cause marked stimulation of the RES 2 days later (4). The fast rate of LDH clearance, studied at this time, was significantly greater in the stilbestrol-treated mice (Fig. 6).

Clearance of Other Enzymes in Infected Mice.—The plasma enzyme increases observable in mice after blockade of the RES or Riley virus infection are of a

specific pattern, some enzymes increasing in activity while others remain unaffected (12). On this basis it was suggested (4) that only those enzymes which are normally cleared by the RES are raised in Riley virus infection. It is clearly of considerable interest to know whether the clearance rates of enzymes other than LDH are altered during infection. We therefore studied in both normal and infected mice the rates of clearance of intravenously injected phosphoglucose isomerase and alanine transaminase. The former enzyme activity is raised about tenfold in infected mice, while the activity of the latter enzyme

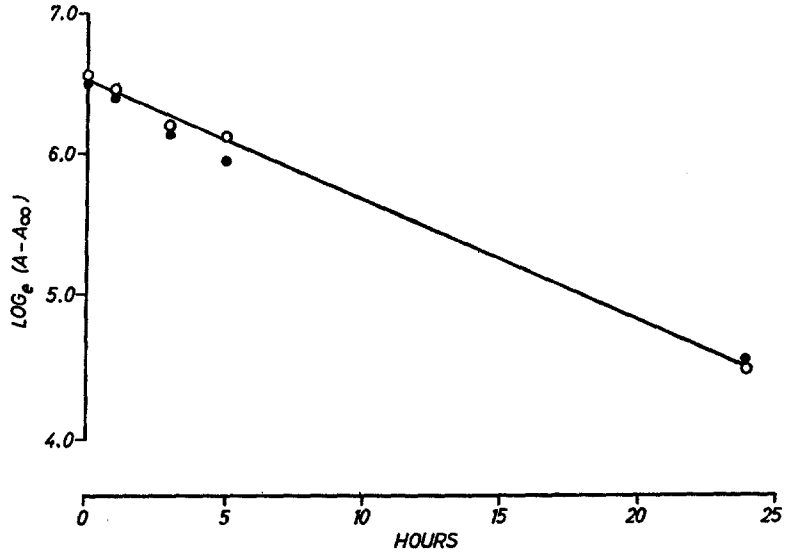

FIG. 8. Clearance of alanine transaminase from the plasma after intravenous injection of 800 IU/liter into normal mice (●——●) and mice infected 7 days previously with Riley virus (O——O). Each point represents a mean value obtained from six mice.

is unchanged (12). There was a very marked impairment of phosphoglucose isomerase clearance in infected mice (Fig. 7), but alanine transaminase clearance was unaltered (Fig. 8). No evidence of fast and slow clearance mechanisms was observed with either of these enzymes over the period studied.

DISCUSSION

It is clear that a basic difficulty associated with enzyme clearance studies is the lack of precise knowledge of the fate of injected enzymes. Many clearance studies have been carried out using radioactively labeled proteins. In some of these the rate of elimination of label from the whole body has been followed (13) while in others the labeled protein has been measured in the extravascular fluid (14). Wasserman and Mayerson (14) found that in dogs the disappear-

ance of labeled albumin from the plasma followed a biphasic exponential curve. The fast phase was considered to represent mixing with the interstitial fluid and lymph, and the slow phase to represent metabolism of the albumin. Although some radioactivity was present in the urine and bile, this appeared to be associated with breakdown products of the originally injected material. Other workers have confirmed these results, and also the biphasic nature of the protein clearance curve with both albumin and globulin (15–18).

Enzyme clearance studies trace an active group or region of a protein molecule and so the possibility exists that the observed elimination of enzyme activity from the plasma may be due, in part at least, to inactivation in the plasma rather than to movement out of it. However, in clearance experiments using radioactively labeled enzymes, it has been shown that the radioactivity and enzyme activity disappear from the plasma at the same rate (19, 20) suggesting that the injected enzyme is removed from the plasma and not merely inactivated.

If it is assumed that enzymes are cleared from the plasma in a manner similar to radioactively labeled proteins, the fast clearance phase could be due to the injected enzyme leaving the blood during the establishment of an equilibrium between the enzyme concentration in the blood and extravascular fluid, while the slow clearance phase could be due to metabolism of the injected enzyme.

If this mechanism were the basis for the biphasic clearance curve obtained with injected LDH, the plasma enzyme level at which the change from fast to slow clearance rate occurs should vary with the injected dose of enzyme and following repeated enzyme injections. However, in the experiments reported above, there was no evidence of this; in fact, the plasma enzyme level at which the change from fast to slow clearance rate occurred was remarkably constant. This suggests that there is in the normal mouse a threshold level for plasma LDH, above which some emergency rapid clearance mechanism comes into operation. The altered clearance curve obtained in Riley virus–infected mice could result from inhibition of this fast clearance mechanism. However, there is no reason why this action alone should cause abnormally high plasma enzyme levels in Riley virus–infected mice which have not been injected with enzyme, unless there is also increased endogenous enzyme production in the infected mice.

The slope of the enzyme clearance curve in Riley virus–infected mice is closely similar to that of the slow phase in normal mice. Now if this slow clearance rate is due to metabolism of the injected protein as has been suggested, it would appear that Riley virus has no effect on the metabolism of injected enzymes. In support of this view, Notkins and his colleagues (21) have shown that the catabolism of radioactively labeled globulin occurs at the same rate in normal and infected mice.

Whatever the mechanism of the fast clearance phase, it is inhibited by Riley

virus, and very similar changes are produced by RES-blocking agents. Our previous finding that the injection of RES-blocking agents into mice resulted in elevated plasma LDH levels was open to criticism that the observed effects might be due in part to a liberation of enzyme consequent upon tissue damage by the drugs used, rather than to an inhibition of plasma enzyme clearance alone. The present results show clearly that treatment with RES-blocking agents inhibits the clearance of injected LDH and supports the idea that Riley virus could act in a similar way. Wakim and Fleisher (22) have reported that zymosan-induced blockade of the RES in dogs results in a similar impairment of LDH clearance. In addition they reported an accelerated clearance of aspartate transaminase during the RES-stimulating phase of zymosan treatment. In the present study, an accelerated enzyme clearance was found during

TABLE I

The Clearance of Enzymes from the Plasma of Normal and Riley Virus–Infected Mice

Enzyme	Source	Half-life in the plasma	
		Normal	Virus-infected
		hr	*hr*
LDH*	Rabbit muscle	1.3	6.1
LDH-1‡	Pig heart	7.2	6.0
LDH-5‡	Rabbit muscle	2.0	5.1
Phosphoglucose isomerase§	Yeast	1.8	20.3
Alanine transaminase§	Pig heart	10.0	10.0

* From Mahy, 1964 (5).

‡ From Mahy and Rowson, 1965 (8).

§ From present paper.

stimulation of the RES with stilbestrol, and it is of considerable interest that mice treated with estradiol are reported to clear guinea pig serum L-asparaginase at an accelerated rate compared to untreated mice (23). There now seems little doubt that the RES plays an important role in the clearance of a number of plasma enzymes, but there are wide differences in the clearance rates between enzymes. For example, the half-life of yeast L-asparaginase in mice was found to be less than 30 min, compared to 19 hr for the same enzyme derived from guinea pig serum (23). Similarly, there were wide differences between the half-lives of aspartate transaminase isoenzymes I and II in dogs (22) and between LDH isoenzymes I and V in mice (8). This suggests that some enzymes may be cleared from the plasma in a different way; i.e., not by the RES but by some slower mechanism. The idea that some enzymes such as alanine transaminase are not cleared by the RES is compatible with the report (22) that zymosan did not consistently affect the level of alanine transaminase in dogs. The present finding (Table I) that the enzymes whose clearance was unaf-

fected by Riley virus (alanine transaminase and LDH-1 isoenzyme) were cleared more slowly in normal mice than the enzymes affected by the virus (LDH-5 isoenzyme and phosphoglucose isomerase) supports both the hypothesis that Riley virus acts by inhibiting the clearance of enzymes by the RES, and the idea that the enzymes which the virus does not affect may be cleared by some system other than the RES.

SUMMARY

The plasma clearance of intravenously injected rabbit muscle LDH was studied. In normal mice the clearance followed a biphasic exponential curve comprising an initial fast and subsequent slow phase. Riley virus–infected mice showed only the slow phase of enzyme clearance. The change from fast to slow clearance rate in normal mice appeared to depend upon the level of plasma enzyme activity rather than on the amount of enzyme cleared.

Treatment of mice with RES-blocking agents (cholesterol oleate and carbon) inhibited the fast clearance phase, whereas an RES-stimulating agent (stilbestrol) caused an accelerated rate of enzyme clearance.

Riley virus infection was found to inhibit the clearance of phosphoglucose isomerase, but had no effect on the clearance of alanine transaminase. The activity of the former enzyme is raised in the plasma of infected mice, whereas the activity of the latter enzyme is unaltered.

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BIBLIOGRAPHY

1. Riley, V., F. Lilley, E. Huerto, and D. Bardell. 1960. Transmissible agent associated with 26 types of experimental mouse neoplasms. *Science.* **132**:545.
2. Mahy, B. W. J., C. W. Parr, and K. E. K. Rowson. 1963. Increased plasma isomerase and transaminase activity in mice infected with lactic dehydrogenase-elevating virus. *Nature.* **198**:885.
3. Plagemann, P. G. W., M. Watanabe, and H. E. Swim. 1962. Plasma lactic dehydrogenase-elevating agent of mice: effect on levels of additional enzymes. *Proc. Soc. Exptl. Biol. Med.* **111**:749.
4. Mahy, B. W. J., K. E. K. Rowson, C. W. Parr, and M. H. Salaman. 1965. Studies on the mechanism of action of Riley virus. I. Action of substances affecting the reticuloendothelial system on plasma enzyme levels in mice. *J. Exptl. Med.* **122**:967.
5. Mahy, B. W. J. 1964. Action of Riley's plasma enzyme-elevating virus in mice. *Virology.* **24**:481.
6. Notkins, A. L., and C. Scheele. 1964. Impaired clearance of enzymes in mice infected with the lactic dehydrogenase agent. *J. Nat. Cancer Inst.*, **33**:741.

7. Bailey, J. M., J. Clough, and M. Stearman. 1964. Clearance of plasma enzymes in normal and LDH agent infected mice. *Proc. Soc. Exptl. Biol. Med.* **117**:350.
8. Mahy, B. W. J., and K. E. K. Rowson. 1965. Isoenzymic specificity of impaired clearance in mice infected with Riley virus. *Science.* **149**:756.
9. Salem, H., M. H. Grossman, and D. L. J. Bilbey. 1963. Micro-method for intravenous injection and blood sampling. *J. Pharm. Sci.* **52**:794.
10. Adams, D. H., K. E. K. Rowson, and M. H. Salaman. 1961. The effect of tumours, of leukaemia, and of some viruses associated with them on the plasma lactic dehydrogenase activity of mice. *Brit. J. Cancer.* **15**:860.
11. King, E. J., and D. M. Campbell. 1961. International enzyme units. An attempt at international agreement. *Clin. Chim. Acta.* **6**:301.
12. Mahy, B. W. J., K. E. K. Rowson, M. H. Salaman, and C. W. Parr. 1964. Plasma enzyme levels in virus-infected mice. *Virology.* **23**:528.
13. Sell, S. 1964. Evidence for species' differences in the effect of serum γ-globulin concentration on γ-globulin catabolism. *J. Exptl. Med.* **120**:967.
14. Wasserman, K., and H. S. Mayerson. 1951. Exchange of albumin between plasma and lymph. *Am. J. Physiol.* **165**:15.
15. Berson, S. A. and R. S. Yalow. 1954. The distribution of I^{131} labeled human serum albumin introduced into ascitic fluid: analysis of the kinetics of a three compartment catenary transfer system in man and speculations on possible sites of degradation. *J. Clin. Invest.* **33**:377.
16. Friedman, J. J. 1957. Vascular-extravascular equilibration of radioactive iodinated albumin in mice. *Am. J. Physiol.* **191**:115.
17. Kekki, M., and A. Eisalo. 1964. Turnover of ^{35}S-labelled serum albumin and gamma globulin in the rat: comparison of the resolution of plasma radioactivity curves by graphic means (manually) and by computer. *Ann. Med. Exptl. Biol. Fenniae (Helsinki).* **42**:196.
18. J. H. Humphrey, and A. S. McFarlane. 1954. Rate of elimination of homologous globulins (including antibody) from the circulation. *Biochem. J.* **57**:186.
19. Schapira, F., J. C. Dreyfus, and G. Schapira. 1962. La durée de séjour dans le plasma de l'aldolase chez le lapin: étude a l'aide d'une aldolase marquée a l'iode radioactif. *Rev. Franc. Etudes Clin. Biol.* **7**:829.
20. Massarrat, S. 1965. Enzyme kinetics, half-life and immunological properties of iodine-131-labelled transaminase in pig blood. *Nature.* **206**:508.
21. Notkins, A. L., S. E. Mergenhagen, A. A. Rizzo, C. Scheele, and T. A. Waldmann. 1966. Elevated γ-globulin and increased antibody production in mice infected with lactic dehydrogenase virus. *J. Exptl. Med.* **123**:347.
22. Wakim, K. G., and G. A. Fleisher. 1963. The fate of enzymes in body fluids—an experimental study. IV. Relationship of the reticuloendothelial system to activities and disappearance rates of various enzymes. *J. Lab. Clin. Med.* **61**: 107.
23. Broome, J. D. 1965. Antilymphoma activity of L-asparaginase in vivo: clearance rates of enzyme preparations from guinea-pig serum and yeast in relation to their effect on tumour growth. *J. Nat. Cancer Inst.* **35**:967.