2.4%) and of the newborn animals 6.2% (range 1.9 to 14.1%), a difference not statistically significant for this small series of experiments. In the newborn animals injected with unconjugated pigment, 28.8% of the label was present in the liver (range 8.7 to 44.8%), while only traces of isotope were detected in kidneys and urine, and none in the brain. After administration of conjugated bilirubin, 5.9% (range 5.7 to 6.1%) of the label was recovered in the liver, and traces were recovered in the urine.

Discussion. In newborn guinea pigs, the finding of reduced plasma disappearance and biliary excretion of injected unconjugated C^{14}-bilirubin supports previous reports that *in vitro,* glucuronide formation in neonatal liver is diminished(1). It is evident, however, that in relation to adult animals, the newborns' capacity to handle the pigment is less severely impaired than would have been expected on the basis of the greatly reduced glucuronide formation *in vitro* as assayed with o-aminophenol as substrate(1). In addition to this defect in conjugation, the liver of newborn guinea pigs was found to exhibit a diminished excretory capacity for conjugated C^{14}-bilirubin. A similar but more severe excretory defect had previously been observed in fetal animals(4). Nonetheless, as revealed by the data in Table I, in the one-day-old guinea pigs, comparable amounts of injected conjugated bilirubin were eliminated more efficiently than unconjugated pigment. This suggests that at this stage of development, conjugation and not excretion is the rate-limiting step in the overall handling of bile pigment.

Summary. Biliary excretion of injected unconjugated and conjugated C^{14}-bilirubin was compared in one-day-old and in adult guinea pigs. In relation to adult animals, in the newborn elimination of unconjugated pigment was reduced by more than half and that of conjugated pigment, by one-third. This indicated that in addition to impaired conjugation, the liver of newborn animals exhibits an excretory defect, but the former represents the rate-limiting step in overall pigment metabolism.

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Received October 4, 1963. P.S.E.B.M., 1964, v115.

Isozymic Patterns in Organs of Mice Infected with LDH Agent.* (28938)

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Riley *et al*(3) first called attention to a transmissible agent which caused elevation of the plasma lactic dehydrogenase (LDH) level. This agent, which was associated with transplantable mouse tumors, was shown to be a coincidental contaminant of tumors and readily separable from them. Until recently, the presence of the agent could be detected only by a rise in plasma LDH and some other enzymes, noted later to be elevated concomitantly(5,6). This rise apparently persists for the life time of the animals(11). Except for splenomegaly, no pathologic changes have been reported in organs of infected animals(12). Riley has recently published some evidence that the agent produces a hemolytic anemia and that the source of the rise in

* Supported by Am. Cancer Soc.

plasma LDH is the red cell(11). Since the organ source of the enzyme gives some clue as to the site of action of the agent, it seemed important to seek confirmatory data for Riley's findings. In the present study, isozymic patterns of LDH activity in various organs were compared with the pattern obtained from plasma. It was assumed that the pattern in the organ would be similar to that liberated into the plasma and that there would not be selective liberation of one particular isozyme. In addition isozymic patterns from various organs of infected and control animals were compared for qualitative differences.

It was also thought to be important to determine whether or not infection by the LDH agent caused a quantitative change in LDH activity in various organs. Plagemann *et al* (5) have reported data to indicate that quantitatively there is no change in LDH activity in a number of organs which they tested. Riley, however, has reported that the organs of an infected animal consistently had a lower LDH activity than organs of a control animal(11). Data to resolve this controversy were sought.

Materials and methods. Non-inbred Swiss mice, 2-6 months old, were used. The agent was originally obtained from DBA/2 Jax mice injected with cells from animals with P-1534 transplantable leukemia.† Plasma from these animals was injected intraperitoneally into Swiss mice in 0.05-0.1 ml amounts. For all subsequent experiments, the agent was recovered from plasma of infected Swiss mice. Blood was collected from the retro-orbital venous plexus in heparinized capillary tubes(1). The plasma was separated by centrifugation and analyzed immediately for LDH activity according to the method of Ells(9). The unit of LDH activity is defined as the amount of enzyme catalyzing the reduction of 1 mμM of 2,6-dichloroindophenol sodium per minute per milliliter of plasma.‡ To determine presence of the agent, plasma levels were measured prior to infection and again after 2 days. Animals thus served as their own controls. Only non-hemolyzed plasma was used.

Quantitative measurements of lactic dehydrogenase activity in various organs were carried out on extracts in 0.25 M sucrose. In one experiment one 2-month-old mouse was injected with 0.05 ml of infected plasma intraperitoneally. A control animal was injected similarly with physiological saline. In addition, 3 5-month-old mice and 3 controls were treated with 0.1 ml of plasma. At 48 hours animals were anesthetized with ether and bled from the retro-orbital plexus. Organs were then dissected free of adherent connective tissue, and blood was rinsed off by brief washing in distilled water. Organs were blotted, weighed and homogenized at 4°C in a glass homogenizer with a Teflon pestle. A determined amount of 0.25 M sucrose was added depending on the expected LDH activity. The homogenate was spun in the Spinco model L preparative ultracentrifuge at 30,000 rpm for 30 minutes at 4°C. The supernatant was diluted appropriately and analyzed for LDH activity using the same procedure indicated above for plasma. The significance of the difference between the means of LDH activity in organs of infected animals *vs* those of control animals was tested using the formula for unpaired variates(8). Student's t distribution was assumed. $P<0.05$ is taken to indicate a statistically significant difference.

For determination of isozymic patterns, one infected mouse with a plasma LDH of 906 units and a control with a level of 136 units were used. Organs were extracted with 0.9% saline and then homogenized and centrifuged as above. Extracts were subjected to electrophoresis in 1% agar gel on 3 × 1″ microscope slides. The procedure was similar to that described by Wieme(4). It was found that only 4 isozymes could be regularly separated using a veronal buffer, pH 8.6, ionic strength, 0.1 (Fig. 2, a, b, c). Substitution of

† The mice were a gift from Dr. Eric L. Simmons, Dept. of Medicine, Argonne Research Hosp., USAEC, Univ. of Chicago.

‡ Dr. Ells informs me that he used cuvettes 1.6 cm in diameter rather than 1.9 cm as stated in his paper. I used cuvettes 1 cm in diameter and therefore multiplied his factor 349 by 1.6 to obtain values comparable to his.

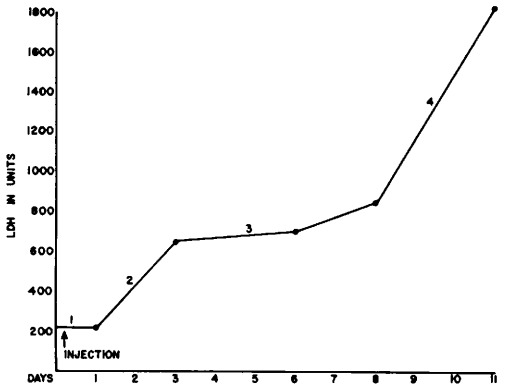

FIG. 1. Plasma LDH values of DBA mice injected with leukemic cells.

a sodium phosphate buffer, pH 7.5, 0.1 M yielded 5 isozymes when electrophoresis was carried out for 2 hours at 250 volts, 10 v/cm. Agar was composed of 1% Difco Special Agar Noble in a solution of phosphate buffer: deionized water, 1:3. The electrode vessels were filled with the phosphate buffer. Approximately 3 lambda of supernatant was introduced into holes punched into the agar. To make the agar adhere better to the slides they were pretreated with a solution containing gelatin, 5 g, and chrome alum, 0.5 g in 1 l distilled water. After electrophoresis, slides were stained immediately for LDH activity according to a modification of the method of Dewey and Conklin(7). The medium contained sodium lactate, 0.1 M; DPN, 0.3 mg/ml; phenazine methosulfate, 0.14 mg/ml; Nitro BT, 2.5 mg/ml (Sigma Co.); sodium cyanide, 0.1 M; and 0.2 M phosphate buffer, pH 7.5. Slides were incubated for one hour in the dark at 37°C. The purple deposits of formazan indicated LDH activity. Control slides were incubated in media lacking substrate or in media lacking DPN and showed no activity. Slides were fixed overnight in a solution of 45% methanol, 10% glacial acetic acid, and 45% water and then dried. Slides were thus transparent and permanent. Control and treated extracts of each particular organ were diluted equally on a weight/volume bases. However, densities of similarly migrating isozymes in different organs are not comparable because no attempt was made to deliver equal units of LDH to the slides.

Results. Fig. 1 illustrates the biphasic rise of activity found to be associated with infection by the agent in the DBA tumor-bearing animals. The first 4 phases of activity noted by Riley(2) are demonstrated. The early rise from 200 units to 650-850 units is related to the LDH agent and occurs before the steep rise associated with tumor growth. The animals were not followed long enough to observe the fall in activity (Phase 5) prior to death. In 24 Swiss mice infected with the agent alone, only phases 1, 2, and 3 were demonstrable. The average LDH level rose in 48 hours from 254 units to 1018 units and persisted at least one month.

Quantitative extraction of organs (Table I) revealed no differences significant at the level of $P<0.05$ at times when plasma activity was 6.5 times higher in infected animals. Data for liver and heart activity approach significance. Because of the dissimilarity of the isozymic patterns (see below), it is possible to rule out the heart as a source, if the assumption that isozymic components are not

TABLE I. Activity of Extractable LDH in Tissues of Mice With and Without Agent.

	LDH units per mg tissue (wet wt)*					
Tissue	Agent	Control	Degree of freedom	t		P
Kidney	42.7; 15.4; 19.5	35.7; 14.2; 21.4	4	.20	>	.8
Heart	39.7; 37.9; 52.3; 49.8	52.0; 16.6; 21.0; 35.7	6	1.6	>	.1
Pancreas	1.1; 5.1; 3.4; 1.3	2.4; 4.6; 2.9; 2.1	6	.25	>	.8
Spleen	8.3; 20.8; 19.0; 17.9	8.6; 13.9; 21.0; 18.2	6	.28	>	.7
Skeletal muscle	18.2; 25.9; 90.2	31.8; 31.0; 60.3; 28.6	5	.10	>	.9
Lung	2.6; 10.2; 8.8; 17.1	5.6; 9.4; 9.9; 8.2	6	.45	>	.6
Brain	3.7; 7.5; 9.0; 9.8	4.8; 7.7; 12.3; 6.2	6	.133	>	.8
Liver	28.3; 41.1; 39.5; 47.4	39.5; 56.3; 68.5; 49.1	6	1.96	=	.1
Plasma†	867; 893; 1341	190; 131; 154	4	5.66	<	.01

* Each value represents a single animal. † LDH units per ml of plasma.

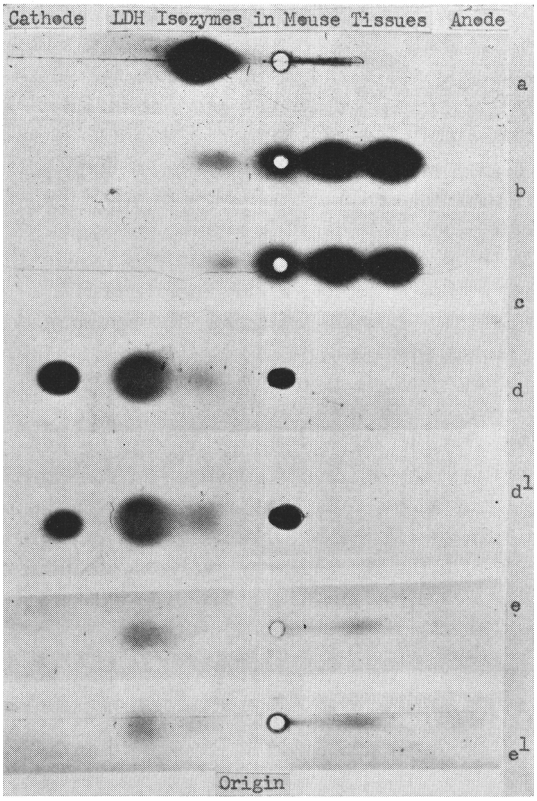

FIG. 2. a. Liver, veronal buffer, pH 8.6. b. Kidney, same buffer. c. Heart, same buffer. d. Red cell hemolysate, infected animal, phosphate buffer, pH 7.5. d^1. Red cell hemolysate, control. The extreme cathodal spot is hemoglobin. LDH at the origin is unexplained. e. Plasma, infected animal, phosphate buffer, pH 7.5. e^1. Plasma, control. Note: The trailing to anodal side of origin in e and e^1 is present in controls also and does not represent LDH activity.

released independently is true. On the basis of the quantitative data alone, the liver remains a possible source. The tendency toward a lower activity in infected liver would indicate that the agent is producing a depletion of enzyme.

Fig. 2, 3 and 4 show that isozymic patterns of organ extracts reveal no qualitative differences between infected and control animals. Three isozymes consistently migrated to the anode and 2 to the cathode. Heart, lung, skeletal muscle, brain and kidney yielded 5 isozymes. Pancreas, spleen, liver, packed blood cells, and plasma yielded only 2 isozymes, the 2 migrating toward the cathode. Despite the fact that quantitative plasma activity appears high in relation to quantitative organ activity, actual units of LDH activity delivered to electrophoresis slides were much greater for most organs than for plasma. It is thus not possible to compare densities of formazan deposits of plasma and organ extracts in Fig. 2, 3 and 4. It should be noted that the cathodally migrating LDH component (LDH5) in mouse red cells is unusual; most mature mammalian red cells have no such component(10).

The plasma pattern thus resembles that of pancreas, spleen, liver, packed blood cells, skeletal muscle, and lung; the last two have a predominance of 2 cathodally migrating isozymes. The quantitative extraction data allow no definite choice to be made among these possible sources although liver must be considered. Because of Riley's demonstration of a hemolytic anemia in infected mice, however, there is reason to select the red cell as a source of plasma LDH. The possibility must remain that other organs having similar isozymic patterns may contribute some activ-

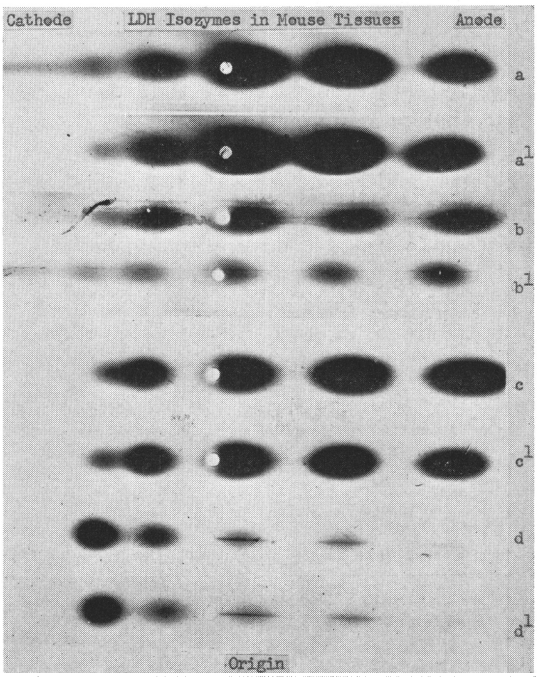

FIG. 3. Phosphate buffer, pH 7.5. a. Heart, infected animal. a^1. Heart, control. b. Brain, infected animal. b^1. Brain, control. c. Kidney, infected animal. c^1. Kidney, control. d. Lung, infected animal. d^1. Lung, control.

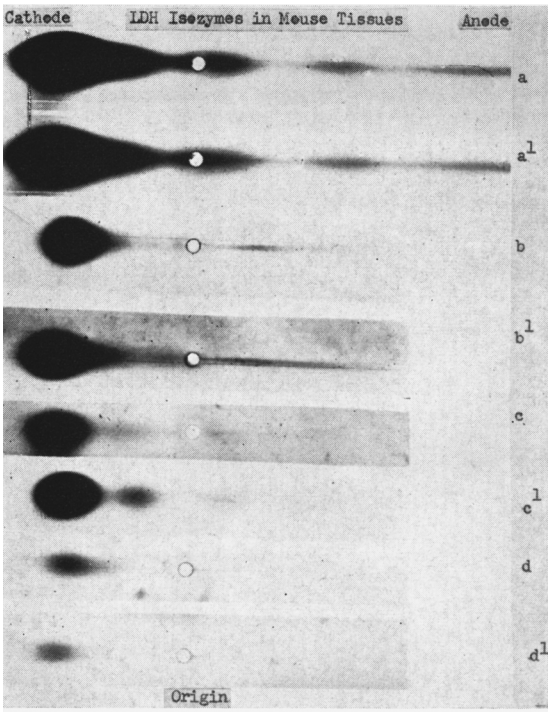

FIG. 4. Phosphate buffer, pH 7.5. a. Skeletal muscle, infected animal. a¹. Skeletal muscle, control. b. Liver, infected animal. b¹. Liver, control. c. Spleen, infected animal. c¹. Spleen, control. d. Pancreas, infected animal. d¹. Pancreas, control.

ity, but other confirmatory data are necessary before accepting them.

Table II gives wet weights for spleens, kidneys, and hearts in 5 infected animals and their controls. The averages are shown; the fraction, infected/control, gives no indication of splenomegaly in these animals. The fact that these animals were killed 2 to 8 days after infection with the virus may explain the discrepancy between these data and the splenomegaly found by Riley since the interval between infection and sacrifice in Riley's animals was 68-138 days.

TABLE II. Organ Weights (g).*

	Infected	Control
Spleen	.164 ± .011	.144 ± .039
Kidney	.548 ± .067	.471 ± .066
Heart	.140 ± .011	.118 ± .025

Infected/control:
Spleen 1.139 Kidney 1.163 Heart 1.186

* Avg, mean ± standard deviation. Each mean is an avg of 5 animals.

The persistence of the agent for long periods with continued elevation of LDH indicates that little or no immunity is developed to the agent. Presence of an infectious agent for prolonged periods makes long-term follow-up of the animals in terms of morphologic changes important.

Summary. The qualitative isozymic pattern of LDH in plasma from Swiss mice infected with LDH agent was found to be similar to that in liver, pancreas, spleen and packed blood cells. It differed from heart, lung, skeletal muscle, brain and kidney. Results of the quantitative analyses of tissue LDH raise the possibility that the liver may contribute to the plasma elevation.

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