Studies on Plasma Lactic Dehydrogenase in Mice with Myeloid Leukemia

II. On the Site of Production of the Enzyme

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SUMMARY

The site of production of the lactic dehydrogenase (LDH) that appears in the plasma of leukemic mice has been studied by technics employing various analogs of nicotinamide adenine dinucleotide. The LDH of several normal organs was characterized and shown to differ from the enzyme which appears in the leukemic plasma. The most likely source of the enzyme, based on these studies, is the tumor cell itself.

This report is part of an initial attempt to study the relation between tumor growth and the appearance of enzymes in the blood plasma. According to Hill and Jordan (3), the concentration of the enzyme, LDH, in the plasma closely followed the growth of a tumor, increasing as the tumor mass increased and decreasing as the tumor regressed. Subsequent studies have confirmed this observation (9).

The accompanying paper (8) correlated the level of LDH[1] in leukemic mice plasma to the severity of the granulocytic leukemia. When the leukemia was in its most advanced state, where not only the bone marrow and spleen but also the liver was diffusely infiltrated by the tumor cells, the level of LDH in the plasma was 14 times normal. Two hypotheses were considered to explain the appearance of LDH at this stage of the disease: (*a*) the volume of tumor reached a critical size such that the rate of LDH production, by the tumor cells, was higher than the normal rate of removal of this enzyme, or (*b*) the liver cells which were displaced by the tumor were ruptured or in some way affected so as to release LDH and other components into the circulatory system. This study describes some substrate specificity characteristics of the LDH from several normal organs of the mouse and an attempt to correlate them to the characteristics of the LDH in leukemic plasma. From these results the first hypothesis is considered more likely —namely, that the LDH appearing at high levels in the leukemic animal is being released from the leukemic tumor cells.

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[1] Nomenclature used conforms to the recent proposal of the Enzyme Commission of the International Union of Biochemistry and of the Biological Chemistry Nomenclature Commission of the Internation Union of Pure and Applied Chemistry (1). Nicotinamide adenine dinucleotide (NAD) is used in place of diphosphopyridine nucleotide (DPN). Analogs of NAD are named according to the substituent replacing either nicotinamide or adenine with the italicized portion referring to the analog part of the coenzyme: acetylpyridine (*AP*), pyridine-3-aldehyde (*PA*), thionicotinamide (*TN*), ethylpyridineketone (*EPK*), 6-hydroxyethylpurine (*6 OHPu*), and hypoxanthine (*Hx*). The abbreviation LDH designates lactic dehydrogenase.

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MATERIALS AND METHODS

The experimental mice were male RF strain, random-bred within the strain, approximately 10 weeks of age. The leukemia was induced by radiation and transmitted to host mice by intravenous injection of a suspension of leukemic spleen cells (8).

NAD and the various analogs were purchased from Pabst Chemical Co., except for *ethylpyridineketone* adenine dinucleotide and nicotinamide *6-hydroxyethylpurine* dinucleotide, which were gifts from Dr. N. O. Kaplan. Sodium lactate was prepared from commercial lactic acid with sodium hydroxide (7).

The various organs were homogenized in 0.2 M sodium phosphate buffer, pH 7.4 (10 ml/gm fresh

weight), in a Potter-Elvehjem-type homogenizer with teflon pestle. The homogenates were centrifuged at 17,000 × *g* for 20 minutes and the supernatants examined.

LDH activity was assayed in both directions at 27°–28° C. First, the oxidation of NADH by pyruvate was measured (8). The second assay was of the reduction of NAD by sodium lactate and was measured in 0.025 M glycine phosphate buffer, pH 10.0, 1 mM NAD or its analog, and 0.10 M or 0.0125 M D,L-sodium lactate and $\frac{1}{20}$ volume of plasma or tissue extract. The pH for the second assay was selected on the basis of a pH dependence curve obtained for a leukemic plasma LDH (Chart 1). The rate of the enzymic catalysis at the two concentrations of lactate was proportional to enzyme concentration at enzyme dilutions which gave an optical density change of less than 0.15 in 5 minutes.

The spectrophotometric constants employed were essentially as compiled by Siegel *et al.* (10); thionicotinamide analog was observed at 395 mμ. Ratios between high and low concentrations of lactate were calculated from the linear portion of the rate curve. In comparing different analogs at a single lactate concentration the observations on NAD, *AP*AD, *APHx*D were made at 350 mμ, and millimolar extinction coefficients of 5.71, 8.08, and 8.12, respectively, were employed. The ratios reported were obtained from reaction rates that were obtained simultaneously, thus eliminating such variables as temperature, enzyme dilution, and aging. An enzyme unit is defined as a change in optical density at 340 mμ of 0.001 per minute at 28° C. by measuring the oxidation of NADH.

Protein was determined by the procedure of Lowry *et al.* (6), with crystalline bovine plasma albumin used as a reference.

RESULTS

Relative rates of reaction with analogs of NAD.—The affinity of LDH for lactate varies depending on the structural configuration of the electron acceptor (5); certain changes in the NAD molecule have more profound effects than others. No attempt has been made to obtain a definitive measure of substrate-enzyme affinity, but in Chart 2 the general relation between the lactate concentration and the rate of its oxidation is illustrated. The comparison of lactic dehydrogenases from various tissues was made in two ways: (*a*) by measuring the ratio of activities obtained at 0.1 M and 0.0125 M lactate with NAD or its analog and (*b*) by measuring the ratio of activities between two analogs at either the high or low concentration of lactate.

In most normal organs of the mouse the rate at the lower concentration of lactate decreased with the series NADH > NAD > N*Hx*D > *APHx*D > *AP*AD > *TN*AD > *PA*AD, with the interchange of *AP*AD and *TNAD* in this series at the higher concentration of lactate. Because of the low rate with *PA*AD, as well as some chemical instability, this analog was less useful. Another analog, *EPK*AD, was observed to undergo chemical reduction at pH 10 but was stable at pH 8.3.

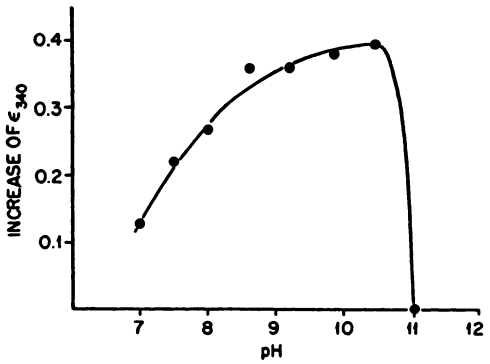

CHART 1.—pH optimum of lactic dehydrogenase in leukemic plasma. Buffers: Tris maleate 0.12 M for pH 7–8.6, sodium aspartate 0.12 M for pH 9.2–11.05. Reaction also contained 0.001 M NAD, 0.025 M sodium lactate, and leukemic plasma containing 10 times the normal lactic dehydrogenase activity.

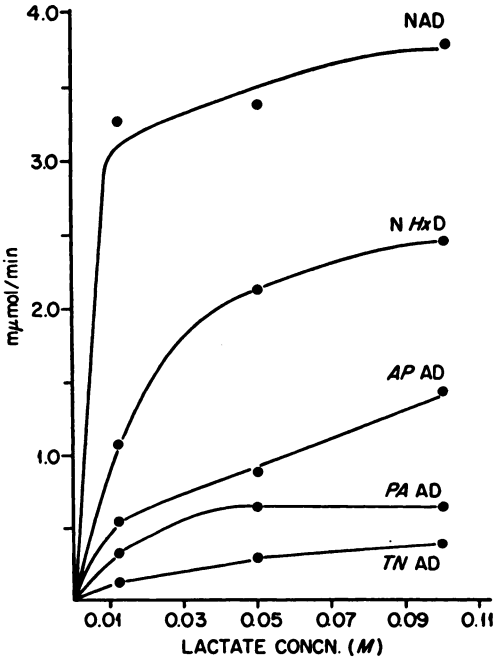

CHART 2.—Substrate concentration vs. rate of heart LDH comparing NAD and analogs. Assay conditions (by NAD reduction) as described in "Methods" except that lactate concentration was varied as shown, and an aliquot extracted from 227 μg. of mouse heart (fresh weight) was used except in the assay of *PA*AD where 4.5 mg. was added.

No measurable reaction occurred in the absence of enzyme between the other analogs and lactate under the conditions of the assay.

Comparison of leukemic plasma with normal organs.—Table 1 shows the LDH characteristics as a ratio of activity of high to low lactate or pyruvate concentration. The leukemic plasma was found to differ from the normal plasma and to resemble that of liver, spleen, muscle, and bone marrow. Distinguishing ratios were found in organs other than these four in the case of one or more analogs. Thymus was differentiated from leukemic plasma on the basis of the *AP*AD and *PA*AD, kidney by four of the six analogs, heart by *AP*AD as well as others, brain by all three values studied, and finally the lymph nodes by three of the analogs employed.

Comparison of leukemic plasma with leukemic and normal organs.—Since the LDH characteristics of several normal organs were indistinguishable from one another as well as from leukemic plasma (Table 1), another series of analog comparisons was performed in which the relative reactivities of a given pair of analogs of NAD were obtained at a given concentration of lactate. In Table 2 the data show that normal liver, spleen, and blood cells can be distinguished from leukemic plasma, whereas the normal muscle enzyme cannot. The normal spleen and liver appear to be similar to each other but different from the leukemic plasma, thus eliminating what was considered a possible source of the enzyme (see introduction). The relative lack of involvement of skeletal muscle in the form of leukemia studied (8) would make this organ an unlikely candidate for the origin of the LDH that appears in conjunction with the leukemic condition. Therefore attention was directed to the tumor cells.

Although the blood was considered as a source of granulocytes, it was not used because technics are not available to prepare granulocytes without contamination by erythrocytes, the latter containing an appreciable level of LDH (12). Since the enlarged spleen of the leukemic mouse is estimated histologically to be composed primarily (80–90 per cent) of the invading leukocytes (8), this organ, in the leukemic condition, was considered the most representative source of the tumor cells. Therefore, extracts were prepared in the usual manner and examined. The results, appearing in Table 2, show a marked correlation of analog ratios between the leukemic plasma and both the leukemic spleen and leukemic liver, thus suggesting the hypothesis that the high levels of LDH result from a release of this enzyme from the tumor cell.

LDH level in normal and leukemic organs.—In addition to the qualitative alteration in the LDH of liver and spleen resulting from the leukemic condition there also occurs a quantitative change most clearly seen in the spleen. Where the spleen is enlarged it is extensively invaded by the tumor cells and, as shown in Table 3, the LDH activity is 1.7–2 times the normal mean value. The plasma LDH values, measured in four of the five leukemic animals referred to in Table 3, were 4,300; 4,900; 24,400; and 40,000 units/ml. The estimated extent of invasion of the liver in these mice was 20, 15, 25, and 60 per cent, respectively; it was 75 per cent in the fifth mouse. Since the spleen has approximately 108,000 units of LDH/gm fresh weight, the values reported in Table 3 probably were unaffected by the LDH contributed from the blood plasma in the organ. From another consideration, also, since the LDH levels in the leukemic livers did not rise, the possible effect of plasma LDH on the spleen values is considered negligible. The slight depression in the liver LDH level (statistically insignificant at the 95 per cent confidence level) may indicate that tumor cells have a lower specific activity of LDH than liver, but this possibility remains to be explored with livers more extensively invaded.

DISCUSSION

The appearance of an abnormally high level of an enzyme in the blood plasma is indicative of the malfunction of certain regulatory mechanisms in the organism. In the case of the high level of LDH which is associated with the terminal stage of granulocytic leukemia in the mouse, there are several possible sites of such malfunction to be considered. First, the behavior of normal tissues in a tumor-bearing animal might be affected in some manner leading to a release and/or increased synthesis of the LDH normally present in the organ, but this possibility is not likely, since the LDH of the normal organs examined had characteristics unlike the leukemic plasma of LDH. There were two exceptions to this conclusion—namely, skeletal muscle and bone marrow. The former is considered as an unlikely source of LDH, since it is relatively uninvolved in this leukemia; with regard to the latter, the bone marrow is probably not the source of the enzyme, since it constitutes a small volume of the animal and has less LDH per unit protein than most organs (unpublished results).

The second possibility to consider is the "factor" or virus reported by Riley (9) which often is associated with transmissible tumors and causes an abnormally high level of LDH in plasma. The data in the accompanying paper (8) did not show the rapid rise in LDH that is reported to occur after

TABLE 1

COMPARISON OF LDH OF LEUKEMIC PLASMA WITH NORMAL ORGANS

NAD analogs	Leukemic plasma	Normal plasma	Liver	Spleen	Muscle	Bone marrow	Thymus	Kidney	Heart	Lymph nodes	Blood cells	Brain	Standard deviation
NADH	1.31 (5)		1.03 (2)	1.16 (2)	1.12 (2)	1.11 (1)	1.06 (2)	**0.82** (2)	0.90 (2)	1.11 (2)			0.24
NAD	2.48 (5)	**1.84** (2)	2.52 (3)	2.29 (3)	2.33 (3)	2.32 (3)	2.34 (3)	**1.76** (2)	**2.09** (3)	2.34 (2)	2.28 (4)	**1.57** (1)	0.22
*NHx*D	3.13 (5)	**2.48** (2)	2.91 (3)	2.75 (3)	2.91 (3)	3.00 (3)	2.89 (3)	**2.46** (2)	2.64 (3)	3.56 (2)			0.33
*AP*AD	1.14 (5)	1.08 (2)	1.39 (3)	1.26 (3)	1.12 (3)	1.08 (3)	**1.85** (3)	1.46 (2)	**3.24** (3)	**2.43** (2)		**2.91** (1)	0.34
*PA*AD	2.85 (5)	2.65 (1)	3.07 (3)	2.59 (3)	2.40 (3)	2.65 (2)	**1.89** (3)	2.55 (2)	**1.78** (3)	**4.10** (1)			0.42
*TN*AD	3.70 (5)	2.97 (2)	3.59 (3)	3.86 (3)	3.50 (3)	3.42 (3)	3.53 (3)	**1.76** (2)	3.51 (3)	**4.82** (2)	**4.64** (4)	**2.33** (1)	0.50
*EPK*AD	4.80 (1)		4.75 (1)	3.87 (1)		3.27 (1)							
N*6OHPu*D	2.00 (1)		1.96 (1)	2.50 (1)		2.00 (1)							

Values are the means of the ratios of LDH activities at 0.10 M to 0.0125 M lactate or 3.0×10^{-3} M to 0.3×10^{-3} M pyruvate. Figures in parentheses are the number of animals examined. The standard deviation for all assays on any one analog is shown. The statistical "*t*" test was applied comparing ratios from each organ to corresponding ratios of leukemic plasma, and those which gave a "*t*" value greater than the 5 per cent level are indicated in boldface type.

The LDH values for the leukemic plasma exceeded the upper boundary of the normal level (2000 units/ml) by factors of 4, 9, 10, 25, and 80.

TABLE 2

COMPARISON OF LDH OF LEUKEMIC PLASMA WITH LEUKEMIC AND NORMAL ORGANS

NAD analog	Leukemic plasma	Leukemic liver	Leukemic spleen	Normal liver	Normal spleen	Normal blood cells	Normal muscle	Standard deviation
*AP*AD:NAD	0.099 (7)	0.099 (5)	0.094 (5)	**0.123** (3)	**0.130** (3)	**0.080** (3)	0.090 (3)	0.01
*TN*AD:*AP*AD	0.983 (6)	0.841 (5)	0.954 (5)	**0.717** (3)	**0.780** (3)	**0.755** (3)	0.893 (3)	0.12

Values are the means of the ratios of LDH activities obtained using the analog combination shown at 0.1 M lactate. All leukemic plasma contained over 15 times the normal level of LDH. See Table 1 for statistical considerations.

injection of this virus but rather a strong tendency to rise only in the terminal stage of the leukemia. Therefore, it is not likely that the LDH virus (9) provides the explanation for the increase in LDH observed in the present study, but this suggestion does not pertain to the possible viral etiology for the leukemia process itself.

The third possibility is that the tumor cells, upon invading an organ, cause the displacement and rupture of normal cells; but again the evidence does not support the hypothesis that the LDH is released from normal cells, whether by destruction of the cell or by malfunction of regulatory mechanisms.

TABLE 3

QUANTITY OF LDH IN NORMAL AND LEUKEMIC ORGANS

Organ	Enzyme activity	
	(Units/mg protein)	(Units/mg fresh weight)
Spleen, normal	797 ± 104*	55 ± 12
Spleen, leukemic	1,368 ± 104	108 ± 12
Liver, normal	2,318 ± 437	208 ± 35
Liver, leukemic	2,078 ± 437	155 ± 35

Units of LDH were determined by the assay involving NAD and 0.1 M lactate and taken from determinations made in Table 2. Three normal and five leukemic organs were examined; the leukemic plasmas (see text) varied by a factor of 10 among themselves, the two lowest being about twice the normal level in plasma.

* Standard deviation.

Finally, the most likely hypothesis remaining is that a variety of materials, including LDH, are released from the tumor cells into the plasma. Supporting evidence in Table 2 shows a similarity of the leukemic plasma LDH to the leukemic liver and spleen enzyme. Incidental to the hypothesis, an increased amount of LDH was observed in the leukemic spleen, indicating that tumor cells have a higher level of LDH than spleen cells. The main unexplained finding is the correlation found between the elevated level of plasma LDH and the diffuse infiltration of the liver. Even prior to this stage, the other organs involved (bone marrow and spleen) have been diffusely infiltrated, but usually no increase in plasma LDH is found (8). The diffuse infiltration of the liver may not be suggestive of a specific liver involvement as much as an indication that the tumor mass, distributed among the various organs, has reached a critical size, and a variety of tumor cell components, including LDH, are released as a result of either an active secretion or cell lysis. Further experiments may be able to distinguish between these two mechanisms.

The existence of several forms of LDH that differ in electrophoretic mobility has been reported (2, 11). The assumption was made in the present study that the various forms of the enzyme would be released impartially and equally and that the substrate affinity characteristics would represent the accumulative characteristics of the individual enzyme forms from a given organ. Finding a high correlation between the LDH of leukemic plasma and of leukemic spleen and liver supports this assumption.

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