MOLECULAR HETEROGENEITY OF LACTIC DEHYDROGENASE IN AVIAN MALARIA (PLASMODIUM LOPHURAE)*

By IRWIN W. SHERMAN

(*From The Rockefeller Institute*)

The establishment and relative importance of the pathways of intermediary metabolism have been the prime concern of earlier biochemical studies of malarial infections. The revealed glycolytic scheme of the host cell, host-parasite complex, as well as that of isolated plasmodia, has shown solely quantitative differences (35). Whether the increments in enzymatic activity are due to the parasite itself, to stimulation of the host cell's metabolism, or to a sum of the activity of the parasite plus the red blood cell, has not been clearly established. Indeed, in view of the recent demonstration by means of electron microscopy that malarial parasites engulf host cell cytoplasm (25, 26), there remains the possibility that even the enzymes which characterize isolated parasites could be those of the erythrocyte.

The nature of such relationships was investigated with regard to the enzyme lactic dehydrogenase (LDH) in white Pekin ducks infected with *Plasmodium lophurae*. LDH catalyzes the reaction pyruvate → lactate in the presence of the coenzyme, reduced diphosphopyridine nucleotide (DPNH), and was particularly well suited for these studies because of the ease of assay by spectrophotometry, relative stability, and the established heterogeneity by a variety of biochemical procedures (5, 6, 8, 12, 15, 16, 21–23, 36–39).

Material and Methods

Enzyme Preparation.—The experimental material was the avian malaria parasite, *P. lophurae*, maintained by the weekly transfer of infected blood in white Pekin ducks. Under the conditions employed, the course of infection is highly synchronous and on the 5th day most parasites are large, multinucleate forms (30). Blood was withdrawn *via* the jugular vein, mixed with one-tenth its volume of physiological saline containing heparin at 27 mg per cent, and the cellular elements separated from the plasma by centrifugation in the cold (4°C) for 10 minutes at 500 g. The plasma was used without further treatment as the enzyme source. Erythrocytes were washed free of other cellular elements by thrice suspending the cells in 5 to 6 times their packed volume of 0.85 per cent (*w/v*) NaCl, recovering the cells by centrifuga-

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tion, and discarding the buffy coat. The soluble enzymes of the erythrocyte, of which LDH is one, were extracted by the addition of an equal volume of 0.1 M phosphate buffer (pH 7.6 ± 0.05) to the packed cell volume (*ca.* 1 to 2 ml), alternate freezing and thawing 3 times in dry ice-alcohol and tepid water baths, centrifugation in the cold at 12,800 *g* for 20 minutes, and withdrawal of the clear, red supernatant by means of a capillary pipette. This 1:1 extract of red cells was diluted 1:49 with phosphate buffer and utilized as another source of the enzyme. Erythrocyte-free *P. lophurae* were prepared by the use of hemolytic serum, trypsin, and deoxyribonuclease as described by Trager (32). The soluble enzymes of these parasite preparations were extracted in a manner similar to that described for erythrocytes. Parasite and red blood cell counts were made in a Petroff-Hauser bacterial counting chamber.

Lactic Dehydrogenase Assays.—Lactic dehydrogenase activity was measured spectrophotometrically in a manner similar to that described by Wroblewski and LaDue (40). To 2.4 ml of 0.1 M phosphate buffer, pH 7.6 ± 0.05, 0.1 ml of DPNH (2.5 mg/ml, Nutritional Biochemicals, Inc., Cleveland) and 0.1 ml of suitably diluted enzyme source (see above) were added. After 10 to 20 minutes at room temperature 0.1 ml of sodium pyruvate (2.5 mg/ml, Nutritional Biochemicals, reagent grade) was added. The optical density decline was followed at 1 minute intervals in a Beckman DU spectrophotometer at 340 mμ for periods of 3 minutes for plasma and starch block eluates (see below), and for 5 minutes for extracts of parasites and erythrocytes. The rate of decrease in optical density represents the rate of oxidation of DPNH to DPN, and a decline in optical density of 0.001 per minute per ml is equal to one LDH unit.

Zone Electrophoresis.—Zone electrophoresis in potato starch has provided a convenient method of separating cellular components and their derivatives. This method, first found to be of value by Vesell and Bearn (36) in establishing the heterogeneity of lactic dehydrogenase activity in serum proteins, seemed to be the most desirable tool since it would combine the advantages of describing inhomogeneities and providing at the same time isolated entities for further analyses. The fractionation method is essentially that of the previously mentioned workers. 1 to 2 ml of 1:1 extracts of cells were separated by zone electrophoresis using a starch medium in barbital buffer, pH 8.6, ionic strength 0.1. The modifications of the Kunkel method (13) have been described in detail (4). We are indebted to Dr. P. A. D'Alesandro for his technical assistance in the use of this technique. Minor variations in our system were: origin 15 cm from the cathode end of the block, use of carbon electrodes, constant voltage of 300 volts for 48 hours, refrigerator temperature 1°C, and elution of 1 cm wide segments with 2 ml of phosphate buffer. Aliquots of the eluates (0.1 ml) were analyzed spectrophotometrically for LDH activity (see above). The distribution of hemoglobin was evaluated by adding aliquots (0.5 ml) of the eluate to 3.0 ml of phosphate buffer and reading the optical density at 540 mμ.

Rapid Kinetic Test.—The rapid kinetic test for determination of heterogeneity of LDH is based on reaction velocities under two selected conditions as described by Plagemann *et al.* (23). The method has as its basis the fact that LDH isozymes (electrophoretically distinct forms of this enzyme) show an inhibition with excess pyruvate, and at a fixed pH each exhibits a substrate optimum. Two concentrations of sodium pyruvate were selected such that at pH 7.0 a high substrate concentration (2.5 μmoles/ml reaction mixture) was optimal for slow LDH and allowed only 43 per cent of the total activity of the fast component. Conversely, a lower concentration of pyruvate (0.59 μmoles/ml reaction mixture) at pH 8.0 was optimal for the fastest and allowed only 44 per cent of the total activity attainable with the slowest. The reaction velocities under these two sets of conditions were designated as v_1 and v_2, respectively, and calculated:

$$v_1 = \text{units slowest LDH} + 0.43 \text{ (units fastest LDH)}$$

$$v_2 = 0.44 \text{ (units slowest LDH)} + \text{units fastest LDH}$$

Since the rates of different isozymes in a mixture are independent of each other and additive, the relative proportion of slow to fast migrating isozymes in a tissue extract can be rapidly estimated by measuring the reaction velocities under the two sets of conditions described, and expressing the result as v_1/v_2. The v_1/v_2 value is relatively constant for a specific tissue and therefore alterations in this figure can be interpreted as either the introduction of a new isozymic component or the elevation of a pre-existing form. It is probably worthwhile to emphasize that temperature, pH, reagent concentrations, and commercial source of DPNH will influence v_1/v_2 values. Therefore, all determinations presented here were made with a single batch of DPNH (Nutritional Biochemicals, No. 5940) and a single batch of sodium pyruvate (Nutritional Biochemicals, No. 9864).

TABLE I

Quantitative and Qualitative Lactic Dehydrogenase Values (Mean ± Standard Deviation) in Avian Malaria

	A Erythrocytes	B P. lophurae	C Plasma
Normal activity v_1/v_2	2.49 ± 0.11 × 10^{-7} units/RBC 0.675 ± 0.041	6.76 ± 3.3 × 10^{-7} units/parasite 0.946 ± 0.031	200*/ml 0.751 ± 0.069‡
Infected activity v_1/v_2	5.39 ± 3.23 × 10^{-7} units/RBC§ 0.827 ± 0.056¶		414 ± 124/ml‖ 0.835 ± 0.125**

* The normal plasma level has been calculated from the chart for 6 to 8 week group so as to compare the values with those of infected plasma. See footnote‖.

‡ Parasitemia = 0 per cent; age 5 to 6 weeks; LDH units/ml = 230 ± 38.

§ Parasitemia = 86.8 ± 27.8.

‖ Parasitemia = 58.8 ± 32.43; age = 6 to 8 weeks.

¶ Parasitemia = 46.7 ± 19.37.

** Parasitemia = 88.48 ± 20.13; age = 5 to 6 weeks; LDH units/ml = 1389 ± 1242.

RESULTS

1. Quantitative Aspects of the Cellular Components.—Erythrocyte LDH activity increased with parasitization (see Table I, column *A*). Variability in LDH quantity, as illustrated by the standard deviations, is shown to be greater for infected than for uninfected erythrocytes. This is taken as a reflection of the variability in percentage of infected cells as well as in the developmental stage of the parasite. The activity of parasitized erythrocytes may be viewed in a somewhat more meaningful manner than that described by its mean when LDH units/ml packed erythrocytes are plotted *versus* parasitemia (parasites per 100 erythrocytes, expressed in per cent) (see Fig. 1 *A*). Such data indicate that the normal red blood cell activity of 9840 ± 2000 was elevated linearly in direct response to parasitemia. The derivation of such a relationship was possible because the infection of ducks with *P. lophurae* was synchronous, and was sampled on the 5th day, when the parasites were large, multinucleate forms. Departures from linearity were sometimes evident, and could be most readily accounted for on the basis of the developmental stage of the plasmodia. Those parasitemias which showed lower LDH activity

than anticipated usually consisted of small, uninucleate trophozoites, whereas parasitemias demonstrating activities higher than those predicted by the curve were generally in a pre-segmenter stage. Erythrocyte LDH activity was thus dependent on the combined factors of parasitemia and parasite growth.
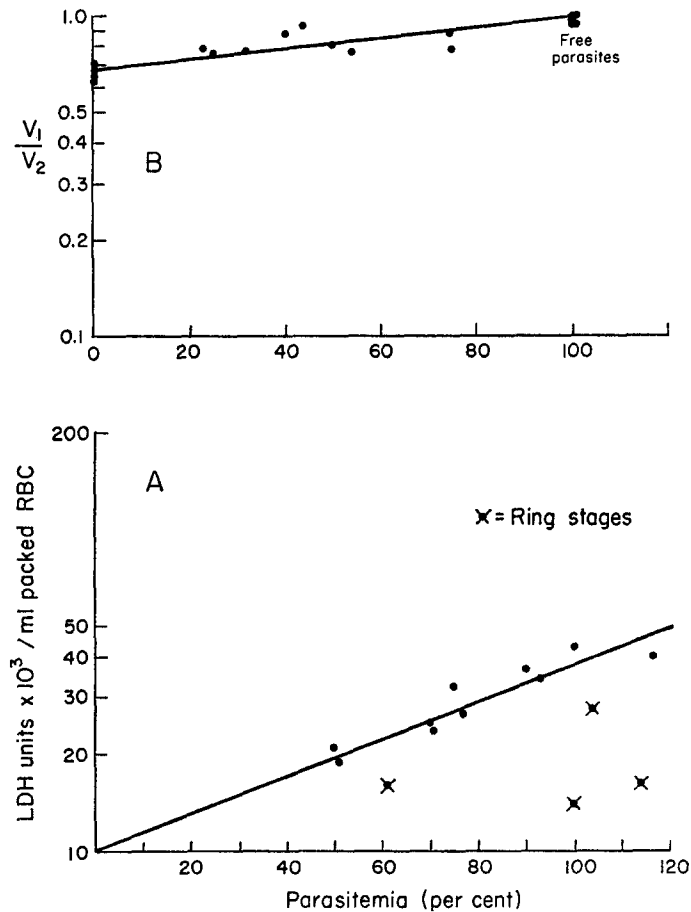

FIG. 1. Lactic dehydrogenase activity and quality in avian malaria. (for explanation see text).

Erythrocyte-free *P. lophurae* had activities which ranged from 1.6 to 5.0 times that of normal red blood cells, and the variability in LDH content in the parasite was again a reflection of its developmental stage. The mean value is shown in Table I, column *B*.

2. Quantitative Aspects of the Plasma.—Duck plasma varied in its LDH content dependent upon the age of the donor (see Fig. 2 *B*). The activity,

like that found for chicken plasma (17), was found to decrease with age, and levelled off at about 10 weeks. In view of this, it was necessary to compare infected and uninfected groups of the same age, or to compare the infected plasma values with the curve of values normally characteristic of the age group. The latter situation is illustrated by the activity value in Table I, column C.
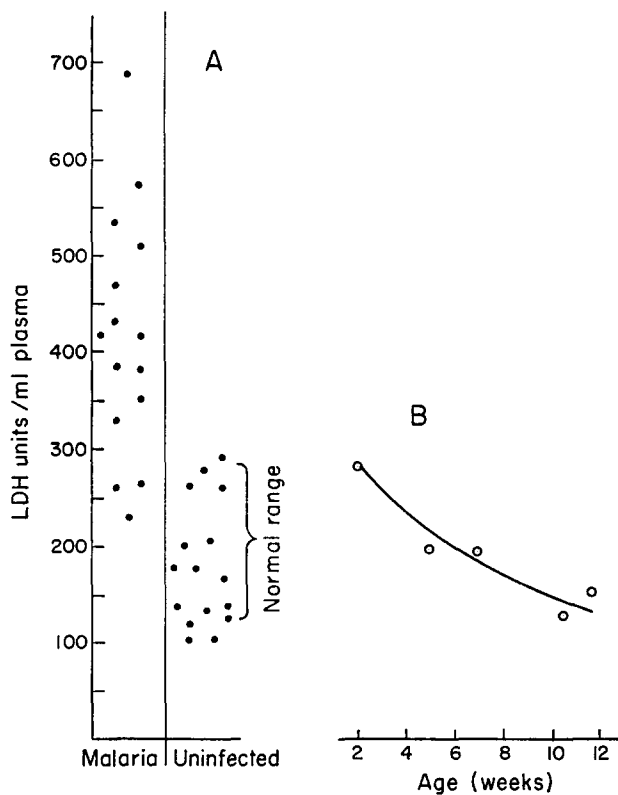

FIG. 2. Lactic dehydrogenase activity in normal and malarial duck plasmas.

The increment in infected plasma LDH level is evident (see Fig. 2 A), but the situation was far more complex than that for infected erythrocytes in that no clear correlation between plasma LDH level and parasitemia appeared. The variability in host response to the malarial infection is well illustrated by the large standard deviations (Table I, column C and footnote**) and by the scatter of points (Fig. 2 A).

3. Qualitative Aspects of the Cellular Components.—

(a) Reaction velocity constants: The data derived from the rapid kinetic test for the heterogeneity of LDH in normal and infected erythrocytes, as well

as for free malaria parasites, are shown as v_1/v_2 values in Table I, columns A and B. The difference between the v_1/v_2 of normal and infected cells is statistically (39) significant ($p = 0.001$). Similar variability existed in the kinetic constants for the various cellular elements as for those described under the quantitative section, and can be explained in the same manner. Since the v_1/v_2 values for infected erythrocytes increased and approached the constant characteristic of $P.$ *lophurae* (Fig. 1 B), it is likely that during the malarial infection there was an increment in the amount of a slow moving LDH.

(b) *Electrophoretic isolation:* As mentioned above, the reaction velocities under the prescribed two sets of conditions predicted the presence of either a new slow moving electrophoretic species, elaborated in response to parasitization and characteristic of the parasite, or more remotely, to the stimulation of pre-existing slow LDH already present in the uninfected red blood cell.

Representative electrophoretic distributions of the various cellular extracts are illustrated in Fig. 3. The results of zone electrophoresis in starch are plotted as percentages of the total recoverable LDH activity, and readily permit comparison of extracts of varying activity. Fig. 3 A clearly shows that the normal duck red blood cells contained a major anodal component (or under the present experimental scheme one which remained at or near the origin), which had 81 to 93 per cent of the recoverable LDH activity in a band 8 cm wide. A minor cathodal band of LDH activity appeared occasionally and was always less than 5 per cent of the total. It may be an artifact. Reticulocytosis (*ca.* 50 per cent reticulocytes produced by intravenous administration of 2 per cent, w/v, phenylhydrazine 2 days prior to sampling) did not alter this pattern. There have been indications that the anodal peak of LDH activity found in normal red blood cell extracts may contain more than one isozyme. However, heterogeneity of the anodal LDH requires further investigation since the possibility of curvature of the migrating front during these long periods of electrophoresis has not been completely ruled out. Extracts of "purified" parasites showed a single, cathodal electrophoretic species which had 75 to 81 per cent of the total activity localized in a band 3 cm wide (see Fig. 3 B). In no case did these extracts show activities characteristic of the normal duck erythrocyte, indicating the high purity of such parasite preparations. Extracts of infected erythrocytes, as anticipated, showed a combination of both these LDH's, and the relative activity in each of the peaks was contingent upon parasitemia and stage of parasite growth (Fig. 3 C).

The distribution of hemoglobin in both infected and uninfected red cells was the same, and confirms the electrophoretic separation of avian hemoglobins previously reported in the literature (11, 27). It should be noted that the parasite extracts contained a very low (<1 per cent) content of hemoglobin, thus illustrating the purity of these preparations as well as substantiating the electron microscopic evidence that parasites contain intact hemoglobin in their food vacuoles.

(c) *pH optimum and Michaelis constant:* The availability of isolated elec-
trophoretic LDH species for both the erythrocyte and parasite prompted two
further lines of investigation. Eluates of 3 to 4 cm of the starch block, contain-
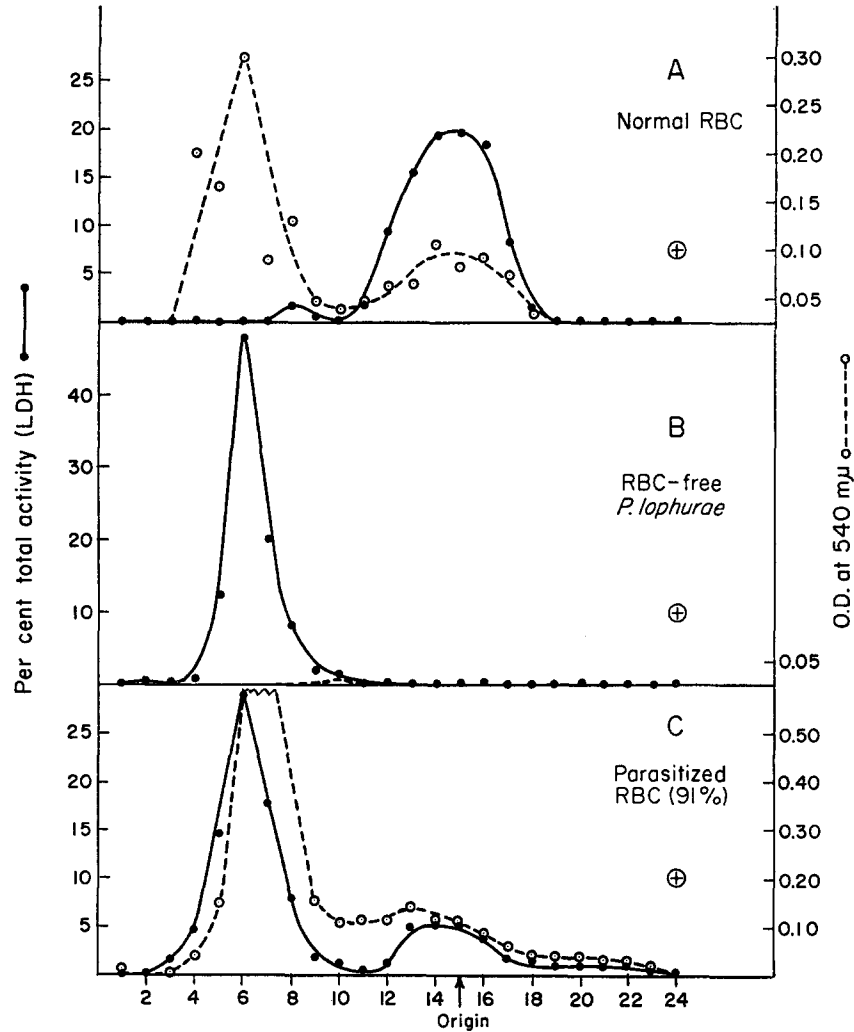

FIG. 3. The electrophoretic distribution of LDH activity in avian malaria.

ing most of the LDH activity, were pooled for the determination of pH optimum
and apparent Michaelis constant (Km) and used as the representative enzyme.
The optimum pH for each was 7.5 (Fig. 4). Although the pH optimum of both
LDH's is the same it should be noted that the parasite enzyme shows distinctly
higher activities than does the erythrocyte enzyme at the extremes of the in-

vestigated pH range. When these LDH's were tested with varying concentrations of sodium pyruvate at pH 7.6 and double-reciprocal plots (Lineweaver and Burk, 14) of the data made, the Km for the red blood cell LDH was 1.7×10^{-5} M, and that for $P.$ $lophurae$ 1.9×10^{-5} M. They are not significantly different.
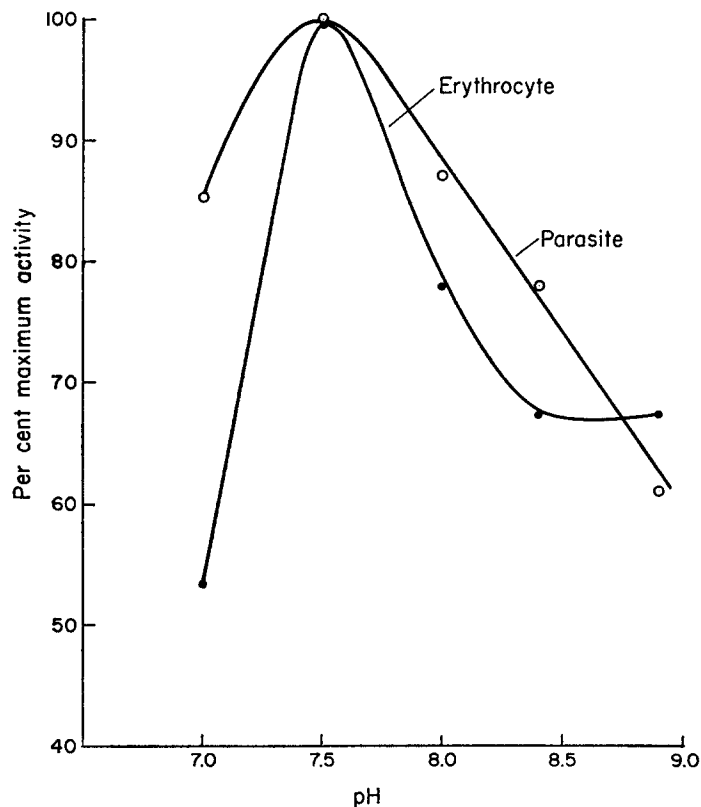

FIG. 4. pH optima of the lactic dehydrogenases of $P.$ $lophurae$ and duck erythrocyte. Reaction mixture contained: 2.4 ml of 0.1 M tris-HCl buffer; 0.1 ml of DPNH (2.5 mg/ml); 0.1 ml of starch block eluate; 0.1 ml of Na pyruvate (2.5 mg/ml).

4. Qualitative Aspects of the Plasma.—The v_1/v_2 values of normal and infected plasmas (Table I, column C) never differed sufficiently to show statistical significance, but infected plasmas did show a trend toward higher values. Since the kinetic constant characteristic of normal plasma was altered in the direction of that found for free $P.$ $lophurae$, it indeed seems likely that the increased activities found in malarious duck plasma are a reflection of the presence of the parasite. This suggestion is further strengthened by the fact

that many plasmas which showed enhanced activity were not colored by hemolysis, and the addition of hemolyzed erythrocytes to the plasma should tend to depress the v_1/v_2 value of plasma. The inability to establish statistical significance for the qualitative alterations in the plasma is probably based on host variability, complicated by interaction of many LDH's from varied cellular sources, especially those of the erythrocyte and the parasite during merozoite liberation which feed into the plasma. These factors, coupled with the lack of predictability of elevation of plasma LDH level, made it seem unreasonable to pursue further studies of a qualitative nature.

DISCUSSION AND CONCLUSIONS

During the last decade there has been witnessed increasing evidence for the heterogeneity of proteins with common catalytic properties, found not only in different organisms, but also within an individual tissue (1). Markert and Møller (16) have termed these isozymes, and they are distinguishable by electrophoretic (6, 16, 22, 36–38), chromatographic (5), coenzyme analog (12), and immunologic methods (8, 21).

Lactic dehydrogenase, one of the first enzymes shown to exist in isozymic forms (19, 20), has retained eminence in the field because of its ubiquity, relative stability, crystallizability, simplicity of assay, and suitability as a clinical index for some pathologic states (9, 10, 24, 28, 36, 37, 39, 40). This enzyme present in freshly isolated malaria parasites as well as infected and uninfected erythrocytes has not been shown to differ in any of these situations and therefore seemed to be a logical choice for investigations of enzymic heterogeneity.

To our knowledge only one other host-parasite complex has been investigated with regard to heterogeneity of enzymes. Bueding and coworkers (8, 15) found the LDH of *Schistosoma mansoni* to differ from that of the rabbit muscle in pH optima, Michaelis constant, and immunologic specificity. In contrast, *P. lophurae* and the duck erythrocyte isozymes show similarly high affinities for pyruvate at pH 7.6 ($Km = ca.$ 1.8×10^{-5} M) and an identical pH optimum (7.5), but molecular heterogeneity could be established on the basis of the v_1/v_2 reaction constants, and zone electrophoresis in starch. This method of demonstrating heterogeneity supports the findings of electrophoretic inhomogeneity of lactic dehydrogenase as first identified by Vesell and Bearn in human serum and red blood cells (36–38).

The kinetic constants (v_1/v_2 of Table I) clearly illustrate that the parasite enzyme is more effective under conditions of low pH and high pyruvate concentration, whereas the erythrocyte enzyme is optimal at a high pH and low concentration of pyruvate. The real significance of these findings comes from the presumed role of LDH in the metabolic economy of each member of the host-parasite complex. Considering LDH as an effective marker of the efficiency of the Embden-Meyerhof scheme the Km and pH optimum values

suggest that both cells are equally equipped to handle carbohydrates glycolytically, and thus derive a similar supply of energy. Paradoxically, the parasite grows at the expense of the host cell, and it does not seem unreasonable to assume that it is deriving more energy for synthesis by turning over more substrate than does the host cell. Thus, as a competitor of the red cell for substrate, the malaria organism appears to have some physiological advantage. Perhaps the answer to this paradox lies in the recognition of the distinction between physiological and *in vitro* enzymatic optima. In the face of presumed enzymic equivalence (based on Km and pH optimum), but observed parasite advantage, the following scheme is proposed. The pH inside the erythrocyte is below 7.5 (2), and the figure probably dwindles to one more acid (*ca.* 7.0) during parasitization owing to excess lactic acid production. Within this pH range the parasite enzyme operates more efficiently than does the red blood cell LDH, a fact supported by the v_1/v_2 value, and by the pH curves (Fig. 4). The pH curves illustrate that at pH 7.0, the physiological state of the erythrocyte, the parasite enzyme is 30 per cent more active. The *P. lophurae* LDH maintains this superiority almost through pH 8.0, although the differences are less striking above pH 7.5. It seems possible that a merozoite, containing small amounts of LDH, but working more actively than a corresponding amount of red blood cell LDH can, in a relatively short time, derive sufficient energy for synthesis of its structural and enzyme proteins. It is of interest to note that Trager (31, 33) has found the cultivation of extracellular *P. lophurae* to be best at pH 6.8–6.9. The striking conclusion of this argument is that the parasite LDH operates more efficiently under the physiological conditions which prevail inside the erythrocyte than does the LDH of the erythrocyte itself.

The electrophoretic LDH of duck erythrocytes resembles that of rabbit erythrocytes (22), save for the possibility of the existence of a minor cathodal LDH in the former. The electrophoretic demonstration of LDH heterogeneity in malaria suggests, although by no means proves, that the enzyme of the erythrocyte and *P. lophurae* differ primarily in their protein structure rather than in the active center.

The present studies in general confirm the quantitative results of McKee *et al.* (18) and it is of interest to note that the calibration curve for *P. lophurae* (Fig. 1 *A*), in magnitude of LDH increase, holds as well for *P. knowlesi* at 50 per cent parasitemia and with 90 per cent of the parasites half-grown or larger. It remains difficult to quantify the LDH content of erythrocyte-free *P. gallinaceum* and *P. lophurae* from the results of Speck *et al.* (29) and Bovarnick *et al.* (3), but it seems quite probable that the bulk of the enzymatic activity observed in their studies was due to the intrinsic metabolism of the parasite rather than to host cell enzyme. Since the 1:1 extracts of erythrocyte-free *P. lophurae* showed less than 1 per cent hemoglobin (presumably as a result of having intact hemoglobin in their food vacuoles), it seems likely that each

parasite must have contained an infinitesimal amount of intact hemoglobin and at the same time little host cell LDH. These quantitative considerations and the morphological evidence seem to warrant the conclusion that the activities observed in free parasites are due, in the main, to their own metabolic machinery rather than to the enzymes of the host cell.

Generally, biological systems illustrate the principle that the whole is greater than the sum of the individual parts, but investigations of LDH in this host-parasite complex demonstrate that the whole equals less than the sum of the parts. The data in Table I indicate that each parasite contains $ca.$ 6.76 $\times$ 10^{-7} LDH units, the normal erythrocyte $ca.$ 2.49 $\times$ 10^{-7} units, and during the course of infection with a mean parasitemia of 86.8 per cent, the average content per erythrocyte was 5.39 $\times$ 10^{-7} units. Simple calculation shows that the theoretical content per red cell should be 8.37 $\times$ 10^{-7} units. The experimental value is $ca.$ 34 per cent less than that anticipated, and can be most plausibly explained by: (a) pluriparasitization of red blood cells which results in stunting of parasite growth and is similarly reflected in depressed parasite enzyme synthesis (see reference 7 for similar interpretation with regard to nitrogen metabolism). Such individuals, although entering into the parasite count would not be up to the general size of isolated parasites; (b) destruction of host cell LDH by phagotrophy, and (c) depressed host cell enzyme synthesis as a result of cell injury. In general, the situation more closely resembles that found for Co (coenzyme) A (32) than the case of folic and folinic acid (34) in $P.$ $lophurae$-infected duck erythrocytes.

These studies have focused attention on the cellular elements, but most clinicians have devoted considerable efforts to investigations of the serological alterations of lactic dehydrogenase activity in a variety of pathological conditions (9, 10, 24, 36–40). There has been, to our knowledge, no such investigation of plasma in malarial infections. Our survey shows rather modest elevations in contrast to neoplastic diseases and such increments seem to be due to "leakage" from the parasite. The disturbing feature in this work has been the lack of uniformity of LDH rise and its rather indistinct qualitative alteration. Perhaps such results are to be expected since the plasma, serving as a reservoir for LDH from a wide variety of tissues is a complex array of isozymes, and qualitative extremes are unlikely. Therefore, plasma LDH quality and quantity seems to be limited in its usefulness as a clinical index in malaria infections.

SUMMARY

Lactic dehydrogenase activity increased in direct proportion to the degree of parasitization in synchronous infections of duck erythrocytes. Deviations from this linearity could be accounted for on the basis of the developmental stage of the parasite. Erythrocyte-free $P.$ $lophurae$ showed activities which averaged 3 times that of uninfected erythrocytes, whereas infected erythrocytes

had intermediate values. In addition, a patent infection was generally reflected by an increase in the lactic dehydrogenase activity in the plasma, but no direct correlation with parasitemia was established.

Molecular heterogeneity of the enzyme was determined on the basis of kinetic data and electrophoretic isolation on a starch block. The uninfected red blood cell showed a major anodal and a minor cathodal peak of lactic dehydrogenase activity, and was further characterized by a kinetic constant representing a high pH optimum with low concentrations of substrate. Isolated *P. lophurae* had a single, cathodal peak of activity dissimilar from that of the uninfected erythrocyte, and a kinetic constant describing a low pH optimum with a high concentration of substrate. Infected erythrocytes showed a combination of these electrophoretic entities and an intermediate range of kinetic constants.

The data indicate that the avian malaria parasite *P. lophurae* contains a lactic dehydrogenase qualitatively dissimilar from that of its host cell, and the increased enzymatic activity of infected erythrocytes is a result of the enzyme content of the growing parasite added to that of the red blood cell. It is suggested that the LDH of the parasite has a physiological advantage under those conditions which prevail inside the red blood cell.

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