The somatic cells of higher vertebrates are known to have at least two structural genes coding for the enzyme lactate dehydrogenase (L-lactate : NAD oxidoreductase, EC 1.1.1.27) (for a recent review of lactate dehydrogenase structure and function see Reference 1). The two genes, Ldh \( H \) and \( Ldh M \), which code for polypeptides \( H \) and \( M \), respectively, are independently regulated; however, if both structural genes are active simultaneously in the same cell, then a total of five enzymatically active tetrameric forms of lactate dehydrogenase may be formed, namely \( H_4 \), \( H_3M \), \( H_2M_2 \), \( HM_3 \), and \( M_4 \), and these can be readily resolved by electrophoresis. The relative proportions of the five isozymes may follow a binomial distribution, indicating completely random hybridization of \( H \) and \( M \) polypeptides into enzymatically active tetramers, or may be nonbinomial, as is the case in many fish (2). Such nonrandom association may arise from the inability of \( H \) and \( M \) subunits in these species to form active tetramers, or perhaps may be due to some form of epigenetic restriction of hybridization (2).

The total complement of \( H \) and \( M \) polypeptides in a given cell type, as determined by their relative rates of synthesis and degradation (3), is felt to be a reflection of the metabolic needs of the particular cell type (1, 4–9). Thus tissues that depend primarily on aerobic metabolism, such as heart tissue or breast muscle tissues in long distance flying birds, have the \( H_4 \) and \( H_3M \) enzymes predominating, whereas tissues such as breast muscle in domestic fowl that must function during short strenuous periods of exercise and hence must be capable of supporting anaerobic glycolysis, have predominantly the \( M_4 \) isozyme (9).
Most tissues, however, are of intermediate metabolic character, and appear to require significant amounts of both polypeptides. In contrast, results from several earlier studies (10-19) suggest that lactate dehydrogenase isozymes of salmonid fish may not follow this general pattern of isozyme distribution. Although quantitative estimation of the levels of H, and M, isozymes in trout has not previously been carried out, preliminary studies suggest that very few tissues in trout are capable of synthesizing or maintaining the M, isozyme (11, 17-19). Similarly, very few cell types, if any, appear capable of simultaneous synthesis or maintenance of H and M polypeptides. This would be quite different from the situation in higher vertebrates in which many cell types synthesize and maintain both H and M polypeptides at varying but significant levels, with the relative amounts of M and H apparently determined by the aerobic nature of the particular cell type (1, 20).

The question arises as to how the salmonid fish's requirement for a balanced complement of lactate dehydrogenase isozymes can be satisfied, if very few tissues have detectable levels of the M, isozyme. We present evidence here that salmonid fish, which have an unusually large number of structural genes for lactate dehydrogenase, may possess a unique and rapidly evolved isozyme system which obviates the necessity in most cell types for simultaneous synthesis and maintenance of the classical M and H polypeptides. Salmonid fish, following an event of total genome duplication, have two genes, Ldh H and Ldh H', synthesizing H and H' polypeptides, both of which are immunologically related to the H subunit of higher vertebrates (11). However, the two polypeptides show differential tissue distribution, and the H'4 isozyme is somewhat intermediate between H4 and M4 in many characteristics, particularly in its key catalytic properties. Further, it is the H and H', rather than H and M, subunits which appear in variable but significant levels in most tissues examined; the M subunit in salmonids was observed in very few tissues. We interpret this to mean that, in salmonid fish the H'4 lactate dehydrogenase is evolving to function catalytically in the absence of a balanced H4-M4 isozyme complement in most tissues. This balance seems to be met in most tissues by H4 and H', rather than H4 and M4.

EXPERIMENTAL PROCEDURE

Materials

Chemicals—Sodium pyruvate, lithium lactate, l(+)-lactic acid, phenazine methosulfate, nitroblue tetrazolium, NAD, and NADH were purchased from Sigma; hydrolyzed starch from Electrostarch Co.; bovine serum albumin from Difco Labs; DEAE-cellulose (DE32) from W. & R. Balston Ltd.; Sephadex G-25, Sephadex G-200, DEAE-Sephadex (A50) from Pharmacia Fine Laboratories, Inc.; and hydroxylapatite from Sigma.

Immunological Reagents—Hemolysin (rabbit, antisheep) and guinea pig complement were purchased from Difco. Sheep red cells in Alsever's solution were a gift from Otago University.

1 It is well known that the unique catalytic, immunological, and physical properties of H and M subunits are maintained even in heterotetrameric enzymes such as H1M, H2M, and H3M. Thus the formation of such heterotetramers is not an obvious prerequisite for cells which require the presence of both H and M subunits; rather, the requirement could be met by an appropriate balance of H1, H2, and H3 homotetramers, even in the complete absence of H1, H2, and H3 heterotetramers. Indeed, such heterotetramers appear to be low in concentration or even totally absent in many species of fish where H1 and M1 exist in a single cell type (2, 21). Alternatively, the apparent absence of such heterotetramers could be an artifact of the electrophoretic separation method (L. M. Maxon and A. C. Wilson, submitted for publication).

Biological Specimens—Quinnat salmon (Oncorhynchus tschawytscha) were obtained through the Marine Department at the Rakia River trapping station in Canterbury, New Zealand. Tissues were stored on Dry Ice for cartage to the laboratory. Brown trout (Salvelinus fontinalis) and brown trout (Salmo trutta) were obtained by conventional angling methods from Otago streams.

**Method**

Assay of Enzymes—For routine assay of lactate dehydrogenase activity, the oxidation of NADH was followed by measuring the rate of decrease in absorbance at 340 nm in a 3-ml reaction mixture containing potassium phosphate (0.1 M, pH 7.5), sodium pyruvate (0.25 M), and lactate dehydrogenase (approximately 0.1 enzyme unit). Absorbance changes at 340 nm were measured with a Beckman spectrophotometer with the Gilford model 2000 recording attachment. All assays were performed at 25°; previous studies showed that the relative catalytic properties of lactate dehydrogenase isozymes from salmon are unchanged if assayed at the fish's preferred temperature of 16°, even though expected absolute changes, such as decreased Km, occurred for all isozymes (15). One unit of enzyme is defined as that amount which causes an initial rate of oxidation of 1 amol of NADH per min at 25°.

For the determination of catalytic constants, a series of assay mixtures were prepared which embraced the approximate range of substrate or product concentrations; 3-ml reaction mixtures containing the enzyme were prepared for each assay, and duplicate assays were performed at each concentration. The data were plotted on both v/s versus v and s/v versus s co-ordinates (v is the net reaction rate and s is the substrate concentration); K and V were determined from the best curve fit to the two plots using a computer program for least squares analysis.

Protein Determination—Protein concentrations were routinely measured at 280 nm, assuming that E1% = 1.0.

Starch Gel Electrophoresis—Electrophoresis was carried out in horizontal gels containing 10% (w/v) starch according to the procedure described by Fine and Costello (22). For gels at pH 6.0, a nitrocellulose phosphatase buffer was used. The buffer was made up as follows: The buffer for the electrode vessels contained 100 ml of citric acid (0.2 M), 320 ml of disodium phosphate (0.2 M), 400 ml of distilled water; the buffer in which the starch gels were prepared contained electrode buffer diluted 3:10 with distilled water. A pH 7.8 buffer system was also used; the electrode buffer contained Tris (0.11 M), borate acid (0.23 M), and EDTA (0.5 mM). The gel was made using a 1:20 dilution of the electrode buffer. Electrophoresis was carried out at 4° with a voltage gradient of about 15 volts per cm for 15 to 20 hours. After electrophoresis the gels were sliced and stained as described by Fine and Costello (22).

Preparation of Searose Extracts of Tissues—Frozen tissues were thawed, minced, and homogenized in a ground glass TenBroek homogenizer with from 1 to 5 volumes of cold 0.25 M sucrose. After centrifugation for 30 min at 30,000 X g, the supernatant containing the enzyme was stored at −10°.

Purification of Heart H4, Lactate Dehydrogenase—Freshly frozen hearts from the quinnat salmon were thawed, washed in cold distilled water, and trimmed of extraneous tissue (228 g). The hearts were homogenized with 3 volumes of cold distilled water for 30 min in a Waring Blender, allowed to stand for 1 hour in the cold, and clarified by centrifugation at 27,000 X g for 30 min. The extract was heated 20 min at 62° followed by quick cooling and centrifugation at 40,000 X g for 30 min. The supernatant was made 40% saturated with ammonium sulfate and allowed to stand overnight at 4°. The mixture was centrifuged to remove insoluble proteins, and ammonium sulfate was added to 60% saturation. The precipitate was harvested by centrifugation and redissolved in the minimum of cold distilled water. Insoluble matter was removed by centrifugation and the solution applied in two runs to a Sephadex G 200 column (1.5 X 85 cm) equilibrated with 50 mM potassium phosphate buffer at pH 7.5. Peak fractions were pooled and reconstituted in 80% ammonium sulfate. The precipitated material was redissolved and extensively dialyzed against 10 mM Tris HCl, pH 7.0, 1 mM mercaptoethanol (Buffer A), and clarified before applying to a DEAE-cellulose column.
Purification of Liver H4, Lactate Dehydrogenase—Salmon liver lactate dehydrogenase was purified as follows. An aqueous extract was prepared from approximately 1.1 kg of pooled livers as described above. The extract was heated to 50°C for 1 min, cooled, and clarified by centrifugation. Ammonium sulfate was added in two stages. Upon reaching 40% saturation, the mixture was centrifugalized to remove inactive protein. At 60% saturation, the bulk of the lactate dehydrogenase was precipitated. The precipitate was dissolved in 2 volumes of ice-cold distilled water, dialyzed against 5 mm Tris-Cl, pH 8.5, and applied to a DEAE-Sephadex A-50 column (3 X 40 cm) equilibrated with the same buffer. A single peak of lactate dehydrogenase activity was eluted from the column in each run and concentrated by precipitation with 70% ammonium sulfate. The peak material was pooled and concentrated by precipitation with 70% ammonium sulfate. The specific activity of the product was 970 enzyme units per mg of protein (over-all purification, 84-fold, yield 5%). Crystallization was carried out by addition of cold saturated ammonium sulfate until a faint precipitate formed. One drop of cold distilled water was added to clarify the solution, which was then allowed to stand at 4°C to allow crystals to grow. Crystals appeared within 4 days.

**Table I**

**Purification of quinnat salmon H4 and H4 lactate dehydrogenases**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>total enzyme</th>
<th>total protein</th>
<th>specific activity</th>
<th>total purification</th>
<th>total yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H4 Isozyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extraction</td>
<td>1.0 X 10^4</td>
<td>2.5 X 10^4</td>
<td>0.024</td>
<td>27</td>
<td>1.0%</td>
</tr>
<tr>
<td>Heat</td>
<td>9.9</td>
<td>4.1</td>
<td>2.3</td>
<td>21</td>
<td>78%</td>
</tr>
<tr>
<td>Amm. sulfate</td>
<td>8.4</td>
<td>1.2</td>
<td>2.9</td>
<td>20</td>
<td>50%</td>
</tr>
<tr>
<td>Sephadex</td>
<td>6.0</td>
<td>0.3</td>
<td>0.065</td>
<td>0.84</td>
<td>4.9%</td>
</tr>
<tr>
<td>First DEAE</td>
<td>4.3</td>
<td>0.35</td>
<td>775</td>
<td>67</td>
<td>34%</td>
</tr>
<tr>
<td>Second DEAE</td>
<td>1.2</td>
<td>0.14</td>
<td>978</td>
<td>78</td>
<td>9.6%</td>
</tr>
<tr>
<td>Third DEAE</td>
<td>0.63</td>
<td>0.065</td>
<td>790</td>
<td>84</td>
<td>4.9%</td>
</tr>
<tr>
<td><strong>H4 Isozyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extraction</td>
<td>23.7</td>
<td>4.35</td>
<td>0.57</td>
<td>1.6</td>
<td>77%</td>
</tr>
<tr>
<td>Heat</td>
<td>18.3</td>
<td>195</td>
<td>0.04</td>
<td>1.6</td>
<td>77%</td>
</tr>
<tr>
<td>Amm. sulfate</td>
<td>13.9</td>
<td>18</td>
<td>0.3</td>
<td>1.1</td>
<td>76%</td>
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<td>DEAE</td>
<td>8.1</td>
<td>0.30</td>
<td>285</td>
<td>499</td>
<td>36%</td>
</tr>
<tr>
<td>Sephadex</td>
<td>7.2</td>
<td>0.14</td>
<td>515</td>
<td>904</td>
<td>33%</td>
</tr>
<tr>
<td>Hydroxyl ap.</td>
<td>2.3</td>
<td>0.024</td>
<td>905</td>
<td>1590</td>
<td>9.6%</td>
</tr>
</tbody>
</table>

Double diffusion and immunoelectrophoresis tests failed to provide evidence for the presence of antibodies to proteins other than lactate dehydrogenase (Fig. 1, A and B), and starch gel electrophoresis showed that the H4 and H4' preparations were substantially free from other isozymes (Fig. 1C). We feel that these preparations of H4 and H4', which are higher in specific activity than those previously reported for salmon isoforms (11, 15), are sufficiently homogeneous that our comparisons of chemical, immunological, and catalytic properties of the two isoforms are valid.

Preparation of Antisera—Antisera were produced in male New Zealand white rabbits. Each rabbit received an initial injection of approximately 0.5 mg of H4 or H4' lactate dehydrogenase, emulsified in Freund's complete adjuvant, subcutaneously at several sites on the back. During the ensuing period of about 3 months an additional 2 mg were administered to each rabbit as two 1-week courses of intravenous injections (without Freund's adjuvant). The rabbits were bled and the sera collected 4 days after completing the second course of intravenous injections. All antisera was stored at −10°C.

**Immunological Techniques**—Double diffusion in agar was performed by a modification of the Ouchterlony method (23) using 15% Davis bacteriological agar buffered at pH 7.5 with 0.1 M potassium phosphate, 0.01 M MgCl2, 0.1 X 10−4 M NaCl, 1.5 X 10−4 M CaCl2, and bovine serum albumin (0.1%) at pH 7.45. The same buffer was used for the thermostat experiments.

Quantitative microcomplement fixation experiments were performed according to the technique of Wasserman and Levine (24). Reaction volumes of 7 ml each were used. The reaction time at 37°C was standardized at 18 hours. The buffer in which all reagents were diluted contained 0.14 M NaCl, 0.01 M Tris hydrochloride, 0.01 M MgCl2, 0.1 X 10−4 M NaCl, and protein was added to appropriate controls with rabbit serum were also carried out to monitor serum enzyme levels.

**Enzyme Precipitation Test**—An enzyme precipitation test was performed by adding antiserum at various concentrations to partially purified muscle lactate dehydrogenase (free from heart isoenzymes). The antiserum was allowed to react overnight (approximately 18 to 24 hours) and the gels were rinsed free of unprecipitated protein and stained with Amido black.

**Molecular Hybridization—** Dissociation and recombination of subunits were achieved by the freeze-thaw technique (20). Prior to use, aliquots of the enzyme solutions were dialyzed against 0.1 M sodium phosphate buffer at pH 8.0 and in the second system the electrode buffer consisted of 0.22 M Tris, 0.45 M boric acid, and 1 M EDTA at a final pH of 7.8. In each case, slides were poured using 1% agar in a 1:3 dilution of the electrode buffer. Electrophoresis was conducted for 1 to 3/4 hours at room temperature at 100 volts and 10 ma per gel for the first buffer system and at 300 volts and 5 ma per gel for the second buffer system. The gels were stained and stained and antiserum was added to the antigen wells. Diffusion at room temperature was allowed to occur overnight (approximately 18 to 24 hours) and the gels were rinsed free of unprecipitated protein and stained with Amido black.

**Enzyme Precipitation Test**—An enzyme precipitation test was performed by adding antiserum at various concentrations to partially purified muscle lactate dehydrogenase (free from heart isoenzymes). The antiserum was allowed to react overnight (approximately 18 hours) in the cold. The antiserum-enzyme preparation was then centrifuged (100,000 X g for 30 min) and the supernatant assayed for remaining enzymatic activity spectrophotometrically as well as on starch gel. Appropriate controls with rabbit serum were also carried out to monitor enzime activity levels.
RESULTS

Tissue Distribution—Starch gel electrophoresis of crude and purified enzymes from the heart and liver of quinnat salmon was performed using several buffer systems. At pH 7.8, five electrophoretically distinct bands can be seen with the heart extract (Fig. 1D). The five bands which are designated H₄, H₄H', H₄H₂', H₄H₃', and H₄ are not all equally intense; the least anodal band, H₄, is by far the most intense and the intermediate bands are sometimes difficult to distinguish. With the liver extract, only a single band is seen (Fig. 1D) which corresponds

Quantitation of Isozymes—Samples were analyzed for isozyme content by applying 10 µl of crude extract, containing a total of 0.02 enzyme unit of lactate dehydrogenase or greater, onto filter paper wicks which were inserted into the gel slots. After electrophoresis overnight, the gels were stained for enzymatic activity for exactly 1 hour at 20°C. Controls were run in which known mixtures of partially purified H₄ and M₄ isozymes, ranging from 100% H₄ to 100% M₄, were subjected to electrophoresis, with a total of 0.6 enzyme unit applied to each gel slot.

Tissue extracts were also diluted in the microcomplement fixation buffer as in the thermostability studies, heated at 55°C for 20 min, cooled, and assayed spectrophotometrically for surviving lactate dehydrogenase activity. As a control, known mixtures of partially purified H₄ and M₄ isozymes, ranging from 100% H₄ to 100% M₄, were subjected to electrophoresis, with a total of 0.6 enzyme unit applied to each gel slot.

For tissues suspected of showing extensive proteolytic activity, namely, pyloric caeca and intestine extracts, a known amount of partially purified H₄ isozyme was added in some experiments to comprise 50% of the total activity in the mixture. After heating, the amount of surviving activity in the mixture was compared to the surviving activity of the heated tissue extract alone, to provide a measure of proteolytic activity.

For tissues suspected of showing extensive proteolytic activity,
exactly in mobility to, and is genetically identical with, the most rapidly migrating band of the heart group, designated the $H_4$ homotetramer.

Although resolution of muscle isoenzymes is poor in the pH 7.8 gel system (Fig. 1, D and E), skeletal muscle can be shown to exhibit a separate set of five isozymes designated $M_4$, $M_4 M'$, $M_4 M''$, $M_4 M'''$, and $M_4'$, all of which appear to be homologous to $M_4$ lactate dehydrogenase of higher vertebrates (11, 15). For simplicity we refer to this set of isozymes in toto as $M_4$ lactate dehydrogenase in this communication. Of the tissue types examined, only three, namely voluntary white muscle, skeletal lateral line tissue (red muscle), and gill filaments show appreciable amounts of $M_4$. The latter two tissues also show high levels of the $H_4$, $H_4'$ isozymes of isoenzymes.

The lateral line and gill extracts also provide evidence that where $M$ and $H$ (or $H'$) polypeptides exist simultaneously in a single cell type in salmon, a reduced, nonbinomial degree of heterotrimer $H$-$M$ hybridization is observed. In vitro dissociation-recombination experiments also result in reduced formation of such hybrid isoenzymes (Fig. 1E). These in vitro hybrid bands were not observed after simple mixing of heart and muscle extracts (Fig. 1E), were produced by in vitro hybridization of the $H_4$, $H_4'$ series with $M_4$ (Fig. 1E), and were precipitable by antisera to $H_4'$ or $H_4$ (not shown). In view of these results we believe it unlikely that the hybrid bands are artifactual. It is interesting to note that recovery of all isozymes was very poor during in vitro hybridization unless NAD was included in the mixture (Fig. 1E); this may account for the previously reported failure to observe in vitro $H$-$M$ hybridization in salmons (17).

All other tissues examined show varying levels of $H$ and $H'$ polypeptides (in the form of enzymatically active tetramers) and of the special $C_4$ isozyme expressed in retinal and neural tissues of some teleosteans (12, 27) or the liver of other teleosteans (28). These tissues have very low or undetectable levels of $M_4$. As shown in Table II, as little as 0.012 enzyme unit of $M_4$ comprising 2% of the total enzyme applied to gel slot, could be readily detected upon electrophoresis in our system, so the apparent absence of $M_4$ isozymes in most tissues examined electrophoretically implies that these tissues indeed have very low levels of $M_4$.

However, several of the tissues examined, such as gallbladder and intestine, have such low total lactate dehydrogenase levels that the maximum amount of enzyme which could be applied to the gel was only 0.02 to 0.10 enzyme unit. Under these conditions, a small but significant (5 to 20%) content of the $M_4$ isozyme may have been overlooked. Therefore an alternative and more quantitative estimation of $M_4$ content was obtained by thermal inactivation of the unstable $M_4$ isozyme. Extracts were heated at 55° under conditions where $M_4$ survival is 0% and $H_4$ and $H_4'$ survival is 100% (Table II). This method employs dilution of the tissue extracts in an albumin-containing buffer, and assumes that the tissue itself has no effect on the stability of the enzyme. In general this was true, but for two of the tissues tested, intestine and pyloric caeca, this assumption breaks down, as proteolytic action is shown to be quite marked (Table II). If, however, the results of both methods are considered, it is apparent that in 11 of the 14 salmon tissue types examined here, the $M_4$ isozyme is a very minor component, probably less than 2%, of the total lactate dehydrogenase complement of the tissue.

**Thermostability**—Studies of lactate dehydrogenases from several organisms including the salmonid fish have shown that different isoenzymes have different distinctive susceptibilities to inactivation by heating. Under the conditions used each isoenzyme has a characteristic rate of temperature inactivation which is independent of the tissue of origin and degree of purity (29). The temperature required for 50% inactivation of lactate dehydrogenase activity in 20 min is designated $T_1/2$ of that isoenzyme.

The susceptibility of purified salmon isoenzymes to inactivation by high temperatures was examined. Fig. 2 shows the
temperature inactivation profile for the purified H₄, H'₄, and M₄ enzymes of salmon. The H₄ homotetramer is more resistant to heat inactivation than the H'₄ enzyme. Tₕ for the H₄ lactate dehydrogenase was approximately 72.5° while the Tₕ for M₄ (the total purification of which will be described elsewhere) was approximately 50°. This is consistent with the values obtained by other workers for unresolved mixtures of trout lactate dehydrogenases (14).

Amino Acid Analysis—The amino acid compositions of highly purified preparations of quinnat salmon H₄, H'₄, and M₄ lactate dehydrogenase are shown in Table III. On the basis of composition alone, the H₄ and H'₄ isoenzymes are clearly closely related in structure and distinct from M₄ in several residues, notably histidine, arginine, aspartic acid, glycine, alanine, and methionine.

Further, the compositional data suggest that H₄ and H'₄ from salmon are not identical in primary sequence. There are significant differences in the number of residues of alanine and isoleucine, and possibly in valine, methionine, and tyrosine in these two proteins. The differences in electrophoretic mobility of H₄ and H'₄, as shown in Fig. 1, are also suggestive of sequence differences. These predictions of sequence differences were therefore tested using sensitive quantitative immunological methods, in which even small differences in immunological properties, and hence presumably in primary amino acid sequence, should be detectable.

Immunological Studies—Ouchterlony double diffusion tests were performed in agar with rabbit antisera against the purified salmon H₄ and H'₄ lactate dehydrogenases. As seen in Fig. 1A, single precipitin lines were obtained in each case. The precipitin line produced by the H₄ enzyme merged with the line produced by the H'₄ enzyme. No spur formation was seen. This would indicate that the two enzymes are immunologically closely related. The absence of secondary lines also provides evidence for the purity of the immunizing preparation, as antisera against crude heart extracts (not shown) are capable of showing six or more precipitin lines when tested against starting material. No cross-reaction could be observed when these antisera were tested against the M₄ preparation.

The microcomplement fixation method has been shown to be sensitive to even very small differences in amino acid sequence (30-33) and capable of providing an approximate quantitative measure of the degree of structural relatedness between homologous proteins (34-36). Antiserum against H'₄ lactate dehydrogenase was tested for reactivity with the purified H₄ and H'₄ isoenzymes. Fig. 3 shows that both the H₄ and H'₄ homotetramers react with the antiserum; at a concentration of 1:7,000, H₄ showed 54% complement fixation, and H'₄ showed 72% fixation. When the anti-H'₄ antiserum was lowered to 1:10,500, then H₄ also showed 54% fixation. The ratio of these two numbers, 1:7,000 + 1:10,500 = 1.50, is called the index of dissimilarity or I.D. The quantity which appears to vary linearly with the percentage sequence difference between related proteins is the immunological distance (33, 34) where

\[
\text{Immunological distance} = 100 \log (\text{I.D.})
\]

Similar immunological distances are obtained using either anti-H₄ or anti-H'₄ antisera (15 and 18, respectively).

The relationship between sequence similarity and immunological cross-reaction cannot be established for lactate dehydrogenases until more sequence information is available. However, from previous studies on the immunological properties of proteins whose sequences are known (33, 37) immunological

---


Table III

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>H₄</th>
<th>H'₄</th>
<th>M₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>26.0 ± 0.7</td>
<td>25.7 ± 0.6</td>
<td>30.0 ± 0.8</td>
</tr>
<tr>
<td>His</td>
<td>8.7 ± 0.8</td>
<td>8.6 ± 0.3</td>
<td>17.6 ± 0.6</td>
</tr>
<tr>
<td>Arg</td>
<td>11.2 ± 0.2</td>
<td>11.4 ± 0.2</td>
<td>8.2 ± 0.7</td>
</tr>
<tr>
<td>Asp</td>
<td>37.7 ± 0.7</td>
<td>35.9 ± 0.6</td>
<td>28.9 ± 0.8</td>
</tr>
<tr>
<td>Thr</td>
<td>17.3 ± 0.6</td>
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<td>17.4 ± 0.6</td>
</tr>
<tr>
<td>Ser</td>
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<td>Glu</td>
<td>28.1 ± 0.9</td>
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<td>21.1 ± 0.6</td>
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<tr>
<td>Val</td>
<td>40.1 ± 1.2</td>
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<tr>
<td>Met</td>
<td>8.8 ± 0.6</td>
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<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>Ile</td>
<td>12.9 ± 0.6</td>
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<tr>
<td>Leu</td>
<td>33.6 ± 2.9</td>
<td>33.8 ± 0.7</td>
<td>36.9 ± 2.4</td>
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<td>Tyr</td>
<td>4.9 ± 0.3</td>
<td>5.9 ± 0.2</td>
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<tr>
<td>Phe</td>
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<td>7.9 ± 0.3</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Trp</td>
<td>4.9 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>5.6 ± 0.4</td>
</tr>
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</table>

Mean ± standard error from duplicate hydrolysates of 24, 48 and 72 hours. Values reported as residues of amino acid per 36,000 grams of protein.

Serine and threonine values extrapolated to zero time of hydrolysis.

Tryptophan determined by direct analysis of hydrolysate containing 4% thioglycollic acid and assuming 80% recovery (50).
distances of this magnitude may represent a difference in amino acid sequence of H and H' in salmon of perhaps 1 to 4%. This is in rough agreement with the amino acid analysis data, which suggest a minimal sequence difference of 9 to 13 residues between the H and H' polypeptides.

As seen from Table IV, salmon H, and H' are about as closely related immunologically as are the H4, H'4 lactate dehydrogenases of chicken and duck, which appear to have separated approximately 100 million years ago.

Tests were also performed here to look for immunological cross-reactivity between H4 and M4 isozymes in salmonids. Using the antisera to salmon H4 and to H'4, we were unable to detect any cross-reaction to salmon or brook trout M4 lactate dehydrogenases, either qualitatively by immunological precipitation of isozymes prior to electrophoresis (13), or immunodiffusion, or quantitatively by the much more sensitive enzyme inhibition and precipitation technique, or by quantitative microcomplement fixation. Some of these results are shown in Fig. 3, A and B, and Table V.

Catalytic Properties—The salmon H'4 and H4 and M4 lactate dehydrogenases were examined for possible functional differences by a comparison of several catalytic parameters. In Fig. 4, A and B, the catalytic reactivities at 25° of the three enzymes at various concentrations of pyruvate and lactate are illustrated. It can be seen that the H4 enzyme has a greater affinity for both substrates and lower optimum substrate concentrations than the M4 enzyme. In addition, the H4 homotetramer is more susceptible to substrate inhibition. These same properties are characteristic of the H, and M4 lactate dehydrogenases of higher vertebrates (9, 11, 20, 28, 39). The H'4 enzyme has an affinity for pyruvate and lactate intermediate between those of the H4 and M4 enzymes with its K_m, optimum substrate concentration, and susceptibility to substrate inhibition being closer to that of the H4 homotetramer than to M4 (Table VI).

It has been proposed that resistance to lactate product inhibition is the most significant functional property of M4 lactate dehydrogenase isoenzymes (39). A study of product inhibition was therefore carried out as suggested by Stambaugh and Post (39). Substrate and product concentrations were selected within values well below the inhibitory levels demonstrated in previous experiments. From results presented in Fig. 5, A to F, and summarized in Table VII, it is clear that product inhibition by lactate (and pyruvate) of the H', enzyme is nearly intermediate between that for the H4 and M4 isozymes. Further, H', is approaching M4 in its substrate optimum and K_m for pyruvate.

The effect of coenzyme (NADH) inhibition on the three isoenzymes was also tested. We observed no significant differences in K_m and optimum concentrations among the three isozymes (Table VI).
Fig. 4. Substrate response of salmon lactate dehydrogenases. A, effect of pyruvate concentration upon the activity of the H4, H'4, and M4 isoenzymes. The initial reaction velocities, computed as the percentage of the maximal rate, are plotted against the concentration of pyruvate. The NADH concentration was 0.14 mM for all determinations. The assay buffer was 0.1 M potassium phosphate, pH 7.5. The assay temperature was maintained at 25°. All points represent triplicate assays. B, effect of L-lactate concentration on the activity of the H4, H'4, and M4 isoenzymes. The initial reaction velocities, computed as the percentage of the maximal rate, are plotted against the concentration of L-lactate. The NAD concentration was 2 mM throughout; the assay buffer was 10 mM Tris-chloride, pH 9.0. The assay temperature was maintained at 25°.

Discussion

Structural and Evolutionary Relationships—Three lines of evidence from earlier studies suggest that salmonid fish have two independently regulated structural genes coding for H4 and H'4 lactate dehydrogenases, both of which are homologous in structure to the well characterized H4 lactate dehydrogenase of higher vertebrates. First, the synthesis and/or breakdown of the H and H' polypeptides in salmonids is independently regulated (11). Also, mutations are known which affect the electrophoretic mobility of the H' subunit in brook trout (10, 15, 19, 40) and rainbow trout (41, 42) but have no effect on the H subunit. Finally, preliminary immunological studies in salmon indicated that H4 and H'4 may be closely related to each other, and both immunologically related to H4 lactate dehydrogenase of higher vertebrates (11, 15). Confirmation and extension of these studies required that pure preparations be made of the H4 and H'4 homotetramers from a single salmonid species.

The results presented here on purified H4 and H'4 lactate dehydrogenase from quinnat salmon demonstrate that these two

Table VI

<table>
<thead>
<tr>
<th>Substrate</th>
<th>H4</th>
<th>H'4</th>
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<tbody>
<tr>
<td>Pyruvate</td>
<td>0.037 ± 0.003</td>
<td>0.075 ± 0.002</td>
</tr>
<tr>
<td>Inhibition</td>
<td>40%</td>
<td>34%</td>
</tr>
<tr>
<td>Lactate</td>
<td>5.8 ± 0.6</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>NADH</td>
<td>0.026 ± 0.001</td>
<td>0.024 ± 0.001</td>
</tr>
</tbody>
</table>

2 Apparent Km and optimum for pyruvate were obtained at 0.14 mM NAD, 25°, pH 7.5, 0.1 M potassium phosphate. Values were computed as described in "Methods", and listed together with computed standard deviations.
3 Apparent Km and optimum for lactate were obtained at 2.0 mM NAD, 25°, pH 9.0, 0.01 M Tris-HCl.
4 Apparent Km and optimum for NADH were measured using 0.33 mM sodium pyruvate, 25°, pH 7.5, 0.1 M potassium phosphate.

Fig. 5. Product inhibition of salmon lactate dehydrogenases. Data is plotted as the reciprocal of the initial reaction velocity (1/v, μmol⁻¹ min⁻¹) versus initial concentration of product in the reaction mixture. A, lactate product inhibition of the purified H4 isozyme at 0.14 mM NAD, 0.1 M potassium phosphate, pH 7.5, 25°, and an initial lactate concentration of (1) 0.1 mM, (2) 0.15 mM, (3) 0.3 mM. B, lactate product inhibition of the purified M4 isozyme; conditions as in A with initial pyruvate concentrations of (1) 0.1 mM, (2) 0.3 mM, (3) 0.6 mM, and (4) 1.0 mM. C, lactate product inhibition of the purified H'4 isozyme; conditions and pyruvate concentrations as in A. D, pyruvate product inhibition of the H4 isozyme at 2 mM NAD, 0.1 M potassium phosphate, pH 7.5, 25°, and initial lactate concentrations of (1) 10 mM, (2) 15 mM, (3) 20 mM. E, pyruvate product inhibition of the H4 isozyme; conditions and initial pyruvate concentrations as in D. F, pyruvate product inhibition of the M4 isozyme; conditions as in D, with initial pyruvate concentrations of (1) 10 mM, (2) 20 mM, (3) 50 mM, (4) 100 mM.
proteins are indeed closely related in structure. The amino acid analysis data suggest that H and H are very similar polypeptides, but that some differences may exist. This prediction was born out by immunological experiments. Immunodiffusion experiments with antisera prepared against purified H and H were incapable of distinguishing the two isozymes; reactions of immunological identity between H and H were observed using this relatively insensitive qualitative technique. However, differences in immunological properties and hence presumably differences in the primary sequence of amino acids of H and H were easily detectable using quantitative microprecipitation fixation. Salmon H and H are about as different from each other immunologically as are the H lactate dehydrogenases of chicken and duck, or the M lactate dehydrogenases of halibut and salmon or halibut and carp.

From the fragmentary fossil record, these orders of birds (36) and superorders of fish (44) diverged between the late Cretaceous and early Tertiary periods, roughly 100 million years ago; thus the duplication event giving rise to H and H would have occurred at this time. Since this duplication event transpired at or very nearly at the time of emergence of the salmonoids (45-48), we would expect to find evidence of a common evolutionary origin of H and M lactate dehydrogenases. The existence of the Ldh H and Ldh H genes in salmonids over such a long span of time, as well as their evolution of independent regulatory capabilities, implies that one of the gene products, either H or H, has evolved an altered and biologically essential function which differs from ordinary H lactate dehydrogenase.

Several additional lines of evidence are presented here which point to the evolution of such an essential and altered function for salmon H lactate dehydrogenase. First, salmon lactate dehydrogenases appear to be unusual in tissue distribution. The H isozyme was observed in only three tissue types: striated muscle, lateral line tissue, and gill tissue. Although very little quantitative information is available on lactate dehydrogenase isozyme ratios in various high vertebrate tissues, most cells previously examined appear to require the presence of significant levels of both H and M subunits in the form of enzymatically active tetramers, to meet a balanced catalytic requirement for lactate dehydrogenase (4-9). In salmonids, it appears to be the H subunit, rather than M, which occurs concomitantly with H in varying amounts in different tissues.

In addition, it is interesting that the H isozyme is intermediate in thermal stability between H and M in salmon, although the relationship between thermal stability and physiological function of lactate dehydrogenases is completely unknown.

But most important to the question of biological function is the catalytic behavior of these isozymes. By a number of catalytic criteria, such as Michaelis constants for pyruvate and lactate, substrate inhibition by pyruvate and lactate, and substrate optima, the H isozyme is somewhat intermediate between H and M from salmon. In particular, the H isozyme shows product inhibition by lactate or pyruvate with inhibition constants (K) almost exactly midway between those for the H and M isozymes. It has been previously suggested (39) that resistance to lactate product inhibition is the crucial catalytic property which accounts for the physiological role of M lactate dehydrogenase. It seems clear that the primary catalytic requirement for H lactate dehydrogenase is that it be able to catalyze the conversion of pyruvate to lactate under relatively anaerobic conditions, even when lactate (product) levels and organisms would imply that the genes for lactate dehydrogenase are diverging much more slowly in salmonid fish than in other vertebrates. This would of course be contrary to the general finding that the over-all rate at which a protein evolves is a reflection of the biological requirements of the protein itself, and is not greatly influenced by the organism or its environment.

**Functional Relationships**—If a gene is duplicated, it is generally assumed (see, for example Reference 43) that one of the duplicated copies must confer a positive selective advantage, such as advantageous increased gene dosage or evolution of a new and advantageous biological function, or else it will be eliminated via incorporation of nonfunctioning mutations. The above information demonstrates that the salmon H and H lactate dehydrogenases are closely related in structure and are the diverging products of a gene duplication event which occurred approximately 100 million years ago. Thus the continuing existence of the Ldh H and Ldh H genes in salmonids over such a long span of time, as well as their evolution of independent regulatory capabilities, implies that one of the gene products, either H or H, has evolved an altered and biologically essential function which differs from ordinary H lactate dehydrogenase.

### Table VII

<table>
<thead>
<tr>
<th>Product inhibition of quininal salmon lactate dehydrogenase isozymes by lactate and pyruvate</th>
<th>Isozyme</th>
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<tbody>
<tr>
<td><strong>Product</strong></td>
<td><strong>H</strong></td>
</tr>
<tr>
<td>Pyruvate Ki (mM)</td>
<td>0.075 ± 0.005</td>
</tr>
<tr>
<td>L-lactate Ki (mM)</td>
<td>28.8 ± 2.3</td>
</tr>
</tbody>
</table>

#### Notes
- Ki values for pyruvate were determined at pH 7.5, 0.1 M potassium phosphate, 20°C, 2 mM NAD, computed as in "Methods" from data in Fig. 4.
- Ki values for lactate were determined as above, with NAD at 0.14 mM.

The immunological results also have direct bearing on important questions raised recently on relatedness between H and M lactate dehydrogenases, and on intrinsic rates of evolution of given proteins in different organisms. Recent studies on the lactate dehydrogenases of brook trout using qualitative immunological methods attempted to demonstrate immunological relatedness between the H and M isozymes in this species (13). Since no cross-reaction between H and M lactate dehydrogenases has ever been reported in other studies of mammalian, reptilian, amphibian, and fish enzymes, such as the finding would provide valuable evidence for the common evolutionary origin of M and H (a point generally assumed but not yet proven). At the same time, however, a demonstration of immunological relatedness of H and M isozymes in salmonid fish but in no other species would imply that the genes for lactate dehydrogenase are diverging much more slowly in salmonid fish than in other vertebrates. This would be contrary to the general finding that the over-all rate at which a protein evolves is a reflection of the biological requirements of the protein itself, and is not greatly influenced by the organism or its environment.

We have at present no explanation for this discrepancy between the two laboratories.
NADH/NAD ratios are high; i.e. this isozyme must show neither lactate product inhibition, nor ability to form a lactate-NADH-enzyme abortive ternary complex at high HADH levels (1). Further, during aerobic utilization of glucose by skeletal muscle, pyruvate must be preferentially converted to acetyl-CoA rather than lactate, and this requires that the $K_m$ for pyruvate of $M_4$ lactate dehydrogenase be greater than that of pyruvate dehydrogenase. That is, a relatively poor substrate affinity for pyruvate is also a very important catalytic parameter of $M_4$ type lactate dehydrogenases. Thus the $H_4$ isozyme in salmonid fish, which is approaching $M_4$ in its resistance to lactate product inhibition and in pyruvate optimum, may be able to function in place of $M_4$ under moderately anaerobic conditions.

The above experimental data suggest that the $H_4$, isozyme in salmon is evolving from a relatively recent gene duplication event, and may satisfy the normal physiological requirement for balanced levels of $H_4$ and $M_4$ lactate dehydrogenases in certain cell types. For reasons which are unclear, these fish appear unable to synthesize or maintain M polypeptides in most cell types; the $H_4$ subunit appears to be evolving to fulfill this need.

At the same time, the $H_4$ isozyme may be similar enough to $H_4$ in certain of its catalytic properties, such as $K_m$ for lactate, to serve as an effective catalyst for lactate scavenging in such tissues as salmon liver, which has only the $H_4$ isozyme. In this sense the $H_4$ isozyme in salmon may be a very generalized lactate dehydrogenase in its functional capabilities.

However, the catalytic properties of $H_4$ suggest that it might not be able to substitute completely for $M_4$ lactate dehydrogenase in certain tissues such as skeletal muscle. For example, rainbow trout under stress have been shown to accumulate lactic acid at scum levels as high as 40 mm (40) and the levels in muscle must be even higher. At such high product levels, the $H_4$ isozyme ($K_i = 53$ mM) would be considerably more inhibited than the $M_4$ isozyme ($K_i = 103$ mM). The $H_4$ isozyme (or $H_4$) thus could function satisfactorily as the sole lactate dehydrogenase enzyme in skeletal muscle of salmon only if total enzyme levels in the tissues were so great that partial inhibition of enzymatic activity during extreme stress would still not severely limit the rate of glycolysis. The $M_4$ isozyme would obviously be capable of providing the required catalytic capacity at considerably lower tissue levels of lactate dehydrogenase than $H_4$ (or $H_4$).

Hence salmonids may have retained a need for the $M_4$ isozyme for balanced levels of $H_4$ and $M_4$ lactate dehydrogenases in certain tissues as salmon liver, which has only the $H_4$ isozyme. In this context, the $H_4$ isozyme (or $H_4$) thus would have proven an obvious selective advantage to the new species. From data presented here, evolution of this isozyme with unique catalytic properties has proceeded extremely rapidly, and with few amino acid changes.

Finally, the in vitro catalytic properties of salmon $H_4$ lactate dehydrogenase reported here are similar to those of higher vertebrate $H_4$ isozymes (1, 39); for an extensive discussion of the metabolic regulation of $H_4$ isozymes via in vivo ternary complex formation, and the relationship of these complexes to catalytic properties measured by conventional in vitro methods see Reference I.

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